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# Regulation of Intestinal Regulatory T cells by Prostaglandin $E_2$

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# Declaration

I declare that this thesis has been submitted by myself, describes my own work except where indicated throughout the thesis, and has not been submitted in any other application for a higher degree.

Siobhan Crittenden

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# Abstract

Pathogenesis of autoimmune and auto-inflammatory diseases is induced by auto-aggressive helper T (Th) cells (i.e. Th1 and Th17 cells), and can be controlled by regulatory T cells (Tregs) characterized by expression of the transcription factor Foxp3. Thus, development of autoimmunity is regulated by the balance of Tregs and Th1/Th17 cells. Prostaglandin $E_2$ ($PGE_2$) is a bioactive lipid mediator with immune-modulatory potential that acts through 4 receptors (EP1-4). It has been shown that $PGE_2$ facilitates Th1 and Th17 cell development and expansion, therefore promoting autoimmune inflammation. However, the role of $PGE_2$ in Treg development and function is largely unclear. The aim of this PhD was to test the hypothesis that $PGE_2$ regulates Treg development, function and subsequent immune response.

I observed that *in vivo* inhibition of endogenous $PGE_2$ biosynthesis using a COX inhibitor resulted in increased $Foxp3^+$ Tregs in various lymphoid organs. This response was prevented by addition of an EP4 agonist. $PGE_2$-EP4 signalling particularly inhibits $ROR\gamma t^+$ Tregs in the intestine. This was not observed in either antibiotic-treated mice or MyD88/TRIF double-knockout mice, suggesting gut commensal microbiota involvement. In addition, $PGE_2$ has a role in microbiota-dependent regulation of intestinal $CD11c^+MHCII^+CD11b^+CD103^-$ mononuclear phagocytes (MNPs) which drive intestinal Treg expansion through production of type 1 interferons. Consistent with these *in vivo* observations, gut microbial metabolites from indomethacin treated mice enhanced *in vitro* $ROR\gamma t^+$ Treg differentiation in the dendritic cell- T cell co-culture system. Adoptive transfer of caecal microbiota from COX inhibitor- treated mice into naïve mice also provided protective benefits in a chemical (DSS)-induced colitis disease model.

In summary, this work has demonstrated that $PGE_2$ affects intestinal Tregs, indicating a novel mechanism for interaction of $PGE_2$, the adaptive immune system and the gut microbiota in homeostasis within this environment. These findings increase our understanding of the role of $PGE_2$ in development of inflammatory bowel disease and offer potential therapeutic strategies for treating this disease.

# Lay abstract

Auto-aggressive T helper cells (Th) cells (i.e. Th1 and Th17 cells) induce autoimmune and autoinflammatory disease pathogenesis, which is controlled by regulatory T cells (Tregs) characterised by expression of the transcription factor Foxp3. The balance of Tregs and Th1/Th17 regulates the development of autoimmunity. Prostaglandin $E_2$ ($PGE_2$) is a lipid mediator with immune-modulatory potential, acting through 4 receptors (EP1-4). It has been shown that $PGE_2$ facilitates Th1 and Th17 cell development and expansion, therefore promotes immune inflammation. However, the effects of $PGE_2$ on Treg development and function is largely unclear.

I have studied this issue and found that $PGE_2$ suppresses intestinal Tregs. While inhibition of endogenous $PGE_2$ using a COX inhibitor increased intestinal Treg numbers, this could be reversed by an EP4 agonist. This effect of $PGE_2$ was not observed in antibiotic-treated mice or MyD88/TRIF double knockout mice, suggesting the involvement of the gut microbiota. I further found that $PGE_2$ suppressed $CD11c^+MHC\text{-}II^+CD103^-CD11b^+$ mononuclear phagocytes (MNPs) which promote intestinal Treg expansion through producing type 1 IFNs. Deficiency of IFNAR prevented COX inhibitor induced enhancement of intestinal Tregs and MNPs. More importantly, adoptive transfer of caecal microbiota from COX inhibitor treated mice into naïve mice reduced chemical (DSS)-induced colitis, which was correlated with increase in Tregs in the host intestine. In addition, while there were comparable Tregs in the gut of T cell-specific EP4 deficient and control mice in steady state, EP4 deficiency in T cells prevented T cell-mediated chronic intestinal inflammation, which also correlated with increase in intestinal Tregs. Taken together, my results indicate a critical impact of $PGE_2$ on intestinal Tregs and the development of intestinal inflammation, possibly through modification of the gut microbiota.

These findings update our understanding of the well-known inflammation mediator, $PGE_2$ in the development of inflammatory bowel disease and offer potential therapeutic strategies for treating this disease.

# List of abbreviations

| | |
|---|---|
| Prostaglandin $E_2$ | $PGE_2$ |
| Hematopoietic stem cells | HSCs |
| Bone marrow | BM |
| Alpha beta | αβ |
| Gamma delta | γδ |
| Cluster of differentiation | CD |
| Double negative | DN |
| T cell receptors | TCRs |
| Major histocompatibility complex | MHC |
| Cortical thymic epithelial cells | cTECs |
| Single positive | SP |
| Antigen presenting cells | APCs |
| Dendritic cells | DCs |
| Pathogen-Associated Molecular Patterns | PAMPs |
| Toll-Like Receptors | TLRs |
| Myeloid differentiation primary response 88 | MyD88 |
| TIR-domain-containing adapter-inducing interferon-β | TRIF |
| Interleukin | IL- |
| T helper cells | Th |
| Transforming growth factor beta | TGF-β |
| Interferon | IFN |
| RAR-related orphan receptor gamma | RORγt |
| Inflammatory bowel disease | IBD |
| Regulatory T cells | Tregs |
| Natural Tregs | nTregs |
| Wild type | WT |
| Forkhead box P3 | Foxp3 |
| Thymic derived Tregs | tTregs |
| Peripherally derived Tregs | pTregs |
| *In vitro* induced Tregs | iTregs |
| Type 1 regulatory T cells | Tr1 |
| Phosphoinositide phospholipase C | PLC |
| Phosphatidylinositide 3-kinase | PI3K |

| | |
|---|---|
| Activin receptor-like kinase 5 | ALK5 |
| Janus tyrosine Kinase | JAK |
| Signal Transducer and Activator of Transcription | STAT |
| Cyclic adenosine monophosphate | cAMP |
| cAMP response element-binding protein | CREB |
| Nuclear factor of activated T cells | NFAT |
| Protein kinase B | AKT |
| Cytotoxic T Lymphocyte Associated Protein-4 | CTLA-4 |
| Indoleamine 2,3-dioxygenase | IDO |
| Gastrointestinal | GI |
| Gut-associated lymphoid tissues | GALT |
| Ovalbumin | OVA |
| Next generational sequencing | NGS |
| Ulcerative colitis | UC |
| Short-chain fatty acids | SCFAs |
| Germ-free | GF |
| Neuropilin-1 | NRP-1 |
| Cytotoxic T-lymphocyte-associated protein-4 | CTLA-4 |
| T conventional cells | Tconv |
| Trinitrobenzenesulfonic acid | TNBS |
| Immature DCs | iDCs |
| Damage associated molecular patterns | DAMPs |
| Pattern recognition receptor | PRR |
| Myeloid differentiation primary response-88 | MyD88 |
| TIR-domain-containing adapter-inducing interferon-β | TRIF |
| Interferon-regulatory factor | IRF |
| Nuclear factor kappa-light-chain-enhancer of activated B | NF-κβ |
| Tumour necrosis factor alpha | TNF-α |
| Inducible T-Cell co-stimulator ligand | ICOSL |
| C-C chemokine receptor type- | CCR- |
| Retinoic acid | RA |
| Intestinal epithelial cells | IECs |
| Retinal dehydrogenases | RALDH |
| Mesenteric lymph nodes | mLNs |

| | |
|---|---|
| Lipopolysaccharide | LPS |
| G-protein coupled receptors | GPCRs |
| Specific pathogen free | SPF |
| TNF receptor associated factor | TRAF |
| TANK binding kinase 1 | TBK1 |
| Suppressor of cytokine signalling | SOCS |
| Genome wide association studies | GWAS |
| Non-steroidal anti-inflammatory drugs | NSAIDS |
| Cyclooxygenase | COX |
| Membrane bound prostaglandin synthase | mPGES |
| Eicosanoid prostanoid | EP |
| Phospholipase A2 | PLA2 |
| Arachidonic acid | AA |
| Prostaglandin | PG |
| Thromboxane | $TXA_2$ |
| Prostaglandin-endoperoxide synthase | PTGS |
| G protein-coupled receptor kinases | GRK |
| Epidermal growth factor receptor | EGFR |
| Adenosine triphosphate | ATP |
| Calcium | $Ca^{2+}$ |
| Fas ligand | FasL |
| 15-hydroxyprostaglandin dehydrogenase | 15-PGDH |
| Collagen induced arthritis | CIA |
| Dextran sodium sulphate | DSS |
| Immunoglobulin | Ig |
| Nicotinamide adenine dinucleotide phosphate | NAPDH |
| Natural killer | NK |
| Hepatocyte growth factor | HGF |
| Mesenchymal stem cells | MSCs |
| Mitogen activated protein | MAP |
| Experimental autoimmune encephalomyelitis | EAE |
| Inducible cAMP early repressor | ICER |
| Innate lymphoid cell | ILC |
| Colony stimulating factor | CSF |

| | |
|---|---|
| Prostaglandin E-major urinary metabolite | PGE-MUM |
| C-reactive protein | CRP |
| Allergic contact dermatitis | ACD |
| Atopic dermatitis | AD |
| Multiple sclerosis | MS |
| Single nucleotide polymorphisms | SNP |
| Ankylosing Spondylitis | AS |
| Caspase-associated recruitment domain | CARD |
| X-box binding protein | XBP1 |
| Lamina propria lymphocytes | LPLs |
| Bone marrow dendritic cells | BMDCs |
| β-mercaptoethanol | B-ME |
| Foetal calf serum | FCS |
| Recombinant human TGF-β | rhTGF-β |
| Phorbol 12-myristate 13-acetate | PMA |
| Phosphate-buffered saline | PBS |
| G-force | g |
| Ammonium-Chloride-Potassium | ACK |
| Ethylenediaminetetraacetic acid | EDTA |
| Room temperature | RT |
| Hanks' Balanced Salt Solution | HBSS |
| Recombinant mouse granulocyte-macrophage colony stimulating factor | GM-CSF |
| Mononuclear phagocytes | MNPs |
| Dimethyl sulfoxide | DMSO |
| Ribonucleic acid | RNA |
| Revolutions per minute | RPM |
| Polymerase chain reaction | PCR |
| Complementary deoxyribonucleic acid | cDNA |
| Glyceraldehyde-3-phosphate dehydrogenase | GAPDH |
| Intraperitoneally | i.p |
| Double knock-out | DKO |
| Interferon receptor | IFNAR |
| Non-small cell lung cancer | NSCLC |

| | |
|---|---|
| Red blood cells | RBCs |
| Yellow fluorescent protein | YFP |
| Green fluorescent protein | GFP |
| Dibutyryl-cAMP | db-cAMP |
| Standard error of the mean | SEM |
| 3-isobutyl-1-methylxanthine | IBMX |
| Lymphocyte antigen 6 complex locus G6D | Ly6G |
| Histone deacetylases | HDAC |
| Aldehyde dehydrogenases | ALDH |
| Myelin basic protein | MBP |
| Interferon-stimulated gene | ISG |
| World Health Organisation | WHO |

# Contents

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# 1 Introduction

## Regulation of intestinal regulatory T cells by prostaglandin $E_2$

The immune system is highly dynamic and has evolved to protect the host organism from endless barrages of both internal potential threats and external pathogens. The immune system includes physical barriers, such as the skin and gut mucosal layer, and the complex cellular and molecular interactions of the innate and adaptive immune responses. The key functions of innate and adaptive immunity are to discriminate between invading antigens or harmless self-antigens and mount an appropriate response [1]. Occasionally the mechanisms in place to recognise our self-antigens as non-threatening can be circumvented, which often leads to the immune system attacking our own tissues resulting in chronic inflammation and autoimmune disease such as Inflammatory Bowel diseases. An autoimmune disease is a chronic condition, but symptoms can vary between mild and debilitating, and having one such condition can increase a person's susceptibly to developing others [2, 3]. These conditions can be treated, but currently not cured. There is no 'specific' treatment for these conditions due to the idiopathic nature of these diseases, but can range between anti-inflammatory drugs such as ibuprofen, immunosuppressive medication and physical therapy [4]. None of these treatments are curative, and can also have equally negative side-effects as explained in the following section. Researching cells that regulate the immune system, such as regulatory T cells and specific molecules that contribute to its function, such as prostaglandin $E_2$ ($PGE_2$), which is known to have both pro- and anti-inflammatory effects, can ensure development of better, more effective treatments.

## 1.1 The Immune System

The immune system consists of multiple components as mentioned above. In addition to physical barriers, multiple cell types are harnessed by the body to protect against both external and internal threats [5, 6].

### 1.1.1 T cell development

Naïve T cells develop within the thymus from hematopoietic stem cells (HSCs) recruited from the bone marrow (BM). There are two distinct thymocyte lineages, alpha beta (αβ) and gamma delta (γδ) [7, 8]. When progenitor T cells first enter the thymus from the BM, they lack the two main surface molecules (cluster of differentiation (CD)4 and CD8) that are associated with mature T cells and are called 'double negative' thymocytes (DN $CD4^-CD8^-$). Cells are negatively selected for those which express T cell receptors (TCRs) which engage with a high affinity for peptides expressed by major histocompatibility complex (MHC)-I or MHC-II proteins on the cortical thymic epithelial cells (cTECs), and those that are unable to engage at all die via apoptosis. Cells are positively selected after TCRs engage with a peptide-MHC molecule complex with a reasonable affinity. The αβ expressing thymocytes represent the majority of the T cell population, and these develop into two distinct subsets; $CD4^+$ and $CD8^+$ T cells [7, 8]. Thymocyte TCRs that bind to MHC-I-peptide complexes mature into $CD8^+$ single positive cells (SP), whereas those that bind to MHC-II-peptide complexes mature into $CD4^+$ SP cells. These cells are classified as mature and exit the thymus to circulate in the blood or lymphatic system.

In this thesis I will be focusing on $CD4^+$ T cells, which shall be explained in further detail in the following sections.

### 1.1.2 Maturation of $CD4^+$ T cells

Development of the adaptive, more specific immune response requires further interaction with cells important in the innate immune system. SP naïve $CD4^+$ T cells exit the thymus and migrate within the lymphoid system prior to activation by antigen presenting cells (APCs). APCs, such as phagocytic dendritic cells (DCs) are part of the first line of defence. These cells process and present proteins on their surface to naïve $CD4^+$ T cell TCRs via the MHC-II complex. In addition to T cell activation by the TCR, co-stimulation of the protein CD28 on the surface of the T cell similarly occurs via the CD80 (B7.1) or CD86 (B7.2) ligands, the combination of which regulates the specificity and strength of the response [9]. The final T cell effector subtype is dependent on the cytokines released by APCs during T cell activation (**Fig. 1.1**) which

is determined by the signal induced by Pathogen-Associated Molecular Patterns (PAMPs) through Toll-Like Receptors (TLRs), vital components of the innate immune response [10]. Following ligation of TLRs with a specific PAMP, a signal cascade is induced through adapter molecules, myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF), inducing cytokine production [1].

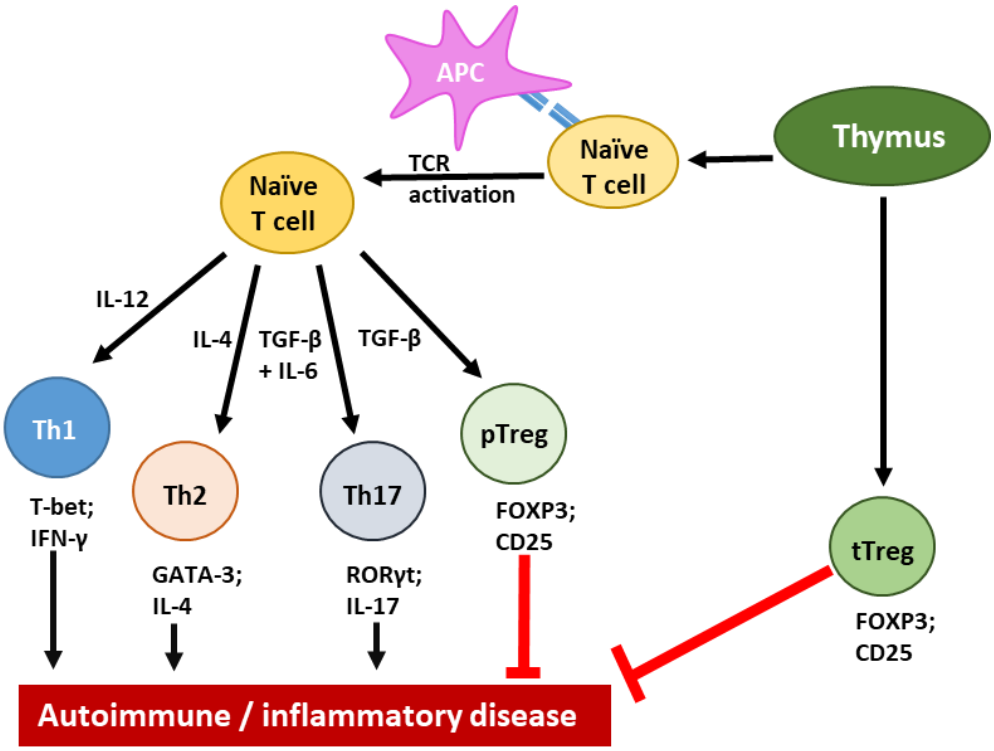


**Figure 1.1: CD4$^+$ Th cell differentiation.** *Naïve T cells migrate from the thymus and are activated by APCs in the peripheral lymphoid organs. In the presence of pro-inflammatory cytokines such as interleukin-12 (IL-12), naïve T cells differentiate into T helper (Th)-1 cells and IL-4 induces Th2 cells. The presence or absence of IL-6 determines whether transforming growth factor (TGF-β) induces either Th17 cells, or peripherally-derived Tregs (pTregs). Thymic-derived Tregs (tTregs) develop within the thymus, then migrate into the peripheral environment. Excessive T helper cell induction can result in autoimmunity and inflammatory diseases, however tTregs and pTregs maintain homeostasis by suppressing their activity [1].*

### 1.1.3 Effector CD4$^+$ T cells

Effector CD4$^+$ T cells are T helper cells (Th1, 2 and 17) which have distinct roles in protecting against potentially harmful pathogens [11]. Th1 cell production of interferon gamma (IFNɣ) is important for the clearance of intracellular pathogens through activation of macrophages and enhanced microbicidal activity [11]. Th2 cells are involved in clearance of extracellular pathogens through cytokine mediated activation of eosinophils, and elevation of B cell derived IgE antibody which is important for clearing pathogens [12]. Th17 cells are thought to be involved in clearance of extracellular bacteria and fungi [13]. Th17 cells secrete IL-17A, IL-17F and IL-22, and are regulated by the transcription factor RAR-related orphan receptor gamma (RORɣt) [14]. Both IFNɣ and IL-4 have been shown to inhibit Th17 differentiation, and IL-4 production by Th2 cells can inhibit Th1 cell induction, suggesting that the Th cells are involved in regulation of each other [15]. Excessive activity of Th2 cells can result in allergies, whereas excessive Th1 and Th17 cells responses can cause autoimmunity development, such as inflammatory bowel disease (IBD) due to aberrant recognition of ‘‘self’’ peptide [15]. The aetiology of this condition is not exact, however it is believed to be due to excessive activation of immune cells against microbial or foreign antigens after disruption to the intestinal barrier [16]. Therefore, it is important to study the mechanism of regulation that prevents this occurring regularly.

### 1.1.4 Regulatory T cells

As described above, self-reactive T cells ‘should’ be eliminated in the thymus, a process known as ‘‘central’’ tolerance. However autoreactive T cells can escape negative selection and be a potential risk if they ever encounter the peptide antigens their TCRs responds to, thus mechanisms to maintain ‘peripheral’ tolerance are also required for a healthy immune system. Regulatory T cells (Tregs) are a subset of CD4$^+$ T cells that suppress the immune response. These cells have been shown to suppress the immune response of mice following immunisation of antigens. In the 1970’s, Gershon and Kondo initially demonstrated the function of ‘suppressor T cells’ by reporting the ability of these cells to suppress an antigen specific immune response, excluding the involvement of B cells and antibodies [3, 17-20].

This finding was expanded by Sakaguchi in the 1990's, when the 'suppressor T cells' were further characterised as $CD4^+CD25^+$ natural regulatory T cells (nTregs) [3]. CD25 is the high affinity receptor for the main cytokine interleukin-2 (IL-2) that promotes T cell proliferation. These nTregs suppressed inflammatory cell responses, and removal of these cells through addition of anti-CD25 antibody (αCD25) to splenocytes from a wild-type (WT) mouse and consequent injection of these nTreg depleted cells into athymic nude mice induced an autoimmune response [3]. The transcription factor and important Treg marker forkhead box P3 (Foxp3) was identified in 2000 as a key regulator of Treg cell development and function [21, 22]. The importance of this protein was demonstrated through use of *scurfy* mice which have deletion in the Foxp3 gene [21, 23]. These mice develop a severe and rapidly fatal lymphoproliferative disease and multiorgan autoimmunity due to inability to control autoreactive $CD4^+$ T cells, however this was relieved by transfer of wild type Tregs [21, 23, 24].

In recent years it has become clear that there are different subsets of Tregs that contribute to immune regulation. Thymic derived Tregs (tTregs), initially termed 'natural' Tregs, are $CD4^+CD25^+Foxp3^+$ T cells that originate in the thymus and are largely self-antigen specific [25, 26]. These represent 5 - 10 % of circulating $CD4^+$ T cells. Peripherally-derived Tregs (pTregs), previously named 'induced/adaptive' Tregs, arise from $CD4^+CD25^-Foxp3^-$ naïve T cells after activation in the peripheral environment by chronic or suboptimal foreign antigenic stimulation, helminth infections and commensal microbiota [25-27]. TGF-β regulates the balance of Tregs or Th17 cells depending on the availability of IL-6 [25]. Tregs generated *in vitro* from naïve T cells are named *in vitro* induced Tregs (iTregs).

There is a subgroup of Tregs that do not express Foxp3, $CD4^+Foxp3^-$ Type 1 regulatory T cells (Tr1). These cells have had increased attention over the past 20 years due to their role in peripheral tolerance, and are activated after long-term activation by an antigen whilst in the presence of IL-10 [28]. In 2013, a panel of surface markers unique for Tr1 cells were discovered including high expression of IL-10 and LAG-3, however there was no unique transcription factor associated with this cell type [28, 29]. These cells also act in specific local environments in response to specific antigens throughout the body similar to pTregs.

The different subgroups shall be referred to as Tregs when discussing the mechanisms of activation and suppression onwards.

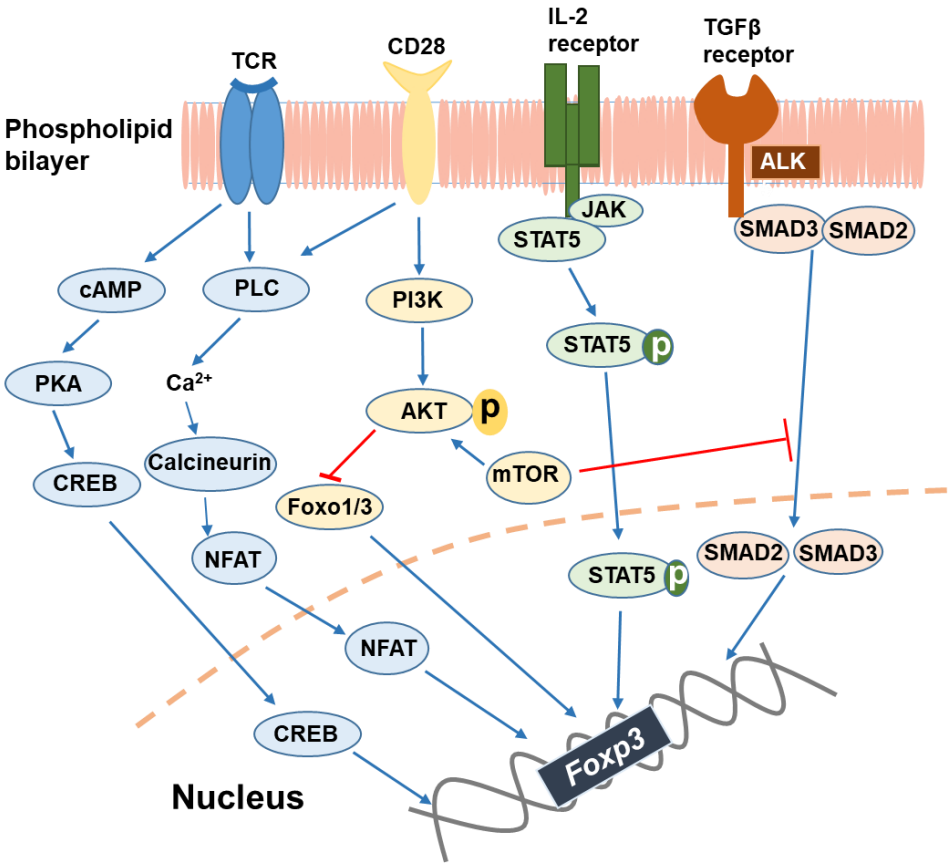


**Figure 1.2: Signalling pathways that mediate Foxp3 induction.** *Multiple signalling pathways, acting via the TGF-β receptor, T cell receptor and IL-2 receptor can result in Foxp3 expression. The CD28 pathway can enhance Foxp3 expression via Phosphoinositide phospholipase C (PLC) signalling, however if the alternate phosphatidylinositide 3-kinase (PI3K) pathway is activated, Foxp3 induction may be inhibited. Image adapted from [30-32].*

Foxp3 expression can be initiated by numerous mechanisms and a combination of TCR activation and cytokine signalling (**Fig. 1.2**). TGF-β is critical for *de novo* induction of Foxp3 expression in T cells through activation of the transcription factor SMAD (i.e. Smad3 and Smad4), via activin receptor-like kinase 5 (ALK5), which bind to the Foxp3 promoter and enhancer to initiate gene expression [30]. IL-2 is another molecule known to be important in the activation and development of Tregs, which acts through its receptor CD25 via stimulation of the Janus tyrosine Kinase (JAK)/ Signal Transducer and Activator of Transcription (STAT)-5 pathway to maintain Foxp3 expression [30].

It was reported that stimulation of the TCR activates the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) which binds to the Foxp3 gene locus and facilitates *Foxp3* gene transcription [30]. However, a recent paper did suggest that CREB deletion in T cells resulted in an increased level of Tregs, and fewer Th17 cells [33, 34]. This is thought to be due to its role balancing Tregs and pro-inflammatory Th17 cells [33, 34]. Phosphoinositide phospholipase C (PLC) can also be activated after TCR stimulation, which acts via calcineurin and nuclear factor of activated T cells (NFAT) for Foxp3 expression [35]. The co-stimulatory molecule CD28 has dual roles with regard to Foxp3 expression. Stimulation can activate PLC to positively up-regulate Foxp3 expression, but can likewise activate phosphatidylinositide 3-kinase (PI3K) and protein kinase B (AKT) [30]. Activation of the PI3K/AKT pathway is thought to prevent *Foxp3* gene transcription [30, 36].

Numerous mechanisms have been proposed as to how Tregs suppress pro-inflammatory $CD4^+$ T helper cells. Tregs can mediate homeostasis through promoting B cell anergy, release of soluble cytokines such as interleukin-(IL)-10 and TGF-β, which can inhibit T cell proliferation and cytokine production [1]. Additionally IL-10 can disrupt DC maturation and APC expression of CD80/86 thus preventing further T cell activation by suppressing co-stimulation [1]. Tregs can similarly act via contact-dependent mechanisms, such as the inhibitory receptor Cytotoxic T Lymphocyte Associated Protein-4 (CTLA-4) which can induce DC production of indoleamine 2,3-dioxygenase (IDO) or granzyme- or perforin-dependent mechanisms which are toxic to responder cells [3].

A balance of these effector T cells and Tregs are vital within the body to prevent infection by harmful antigens, but also to protect against excessive inflammation which could have deleterious effects on tissues and the body. However, there are certain areas of the body which are more likely to encounter potentially pathogenic organisms or material, and thus requires a more active immune system to be present, adapted for this specific environment, such as the cilia, goblet cells, a mucosal layer and the immune cells within the nasal passage, the lungs and intestine. These areas of the body are continuously in contact with the external environment and foreign bodies. The intestine is the tissue which was focused on for this PhD.

## 1.2 Intestinal Immune System

### 1.2.1 Development of intestinal immunity

The gastrointestinal (GI) epithelium at approximately 100 $m^2$ is considered to be the largest immune interface with the environment, and is constantly exposed to non-harmful stimuli, such as microbiota and food particles which normally would trigger an immune response [37]. Therefore, the gut requires sophisticated regulatory mechanisms to prevent an excessive immune response to these non-harmful stimuli [5, 38]. However, protection against harmful pathogens invading the gut is necessary and demonstrates the importance of a strong defence system within this environment. This defence includes non-specific mechanisms including digestive enzymes and pathogen-specific defence provided by the gut-associated lymphoid tissues (GALT) [6, 16, 39]. GALT itself is known to develop under the influence of the non-harmful microbiota and their resulting metabolites [16, 40].

One method of preventing response to foreign proteins in food is known as ‘‘oral tolerance’’. In 1909, Besredka initially observed the effect of dietary antigens such as milk whey inducing oral tolerance, when guinea pigs administered rectally or orally by this antigen were resistant to sensitisation and allergy development after injection of the antigen at a later point [41]. This finding was supported in 1911 by Wells and Osborne who demonstrated that delivering proteins to guinea pigs from the weaning stage prevented allergic reaction development after future injection with the antigen [41]. The importance of T cells in induction of oral tolerance was demonstrated in the 1970’s by experiments in which donor mice were fed with ovalbumin (OVA), and T cell enriched splenocytes from these mice were injected into naïve recipient mice which were resistant to OVA-induced inflammation compared to mice injected with splenocytes depleted of T cells [42]. Further studies examining oral tolerance in the intestine have been carried out using experiments such as depletion or suppression of antigen-specific T cells [42].

It was then questioned whether tTregs or pTregs were more important for this process. Bilate used the process of crossing TCR transgenic mice (in which all the $CD4^+$ T cells recognise OVA) with RAG-1 deficient mice, which produce no mature T or B cells, and injected the OVA antigen to induce an immune response [42, 43]. Oral exposure to the antigen resulted in increased $CD4^+CD25^+Foxp3^+$ pTregs that had a strong suppressive ability both *in vivo* and *in vitro* [43].

Another mode of immune tolerance is directed against the intestinal commensal microbiota. There are approximately 1 x $10^{14}$ gut microbes within the intestine, a combination of bacteria, viruses and fungi [44]. Their combined genetic information encodes 100 times the genes contained within the human genome [45]. The microbiota mostly acts in a symbiotic manner with the host but protection against potential threats is still important. Before birth, an individual's gastrointestinal tract is sterile, but this becomes colonised by the mother's microbiota during delivery [46]. The importance of a diverse gut microbiota is clear, and its composition can be influenced by a multitude of factors. The differences between delivery modes can massively affect microbial diversity. Infants born through caesarean section had a lower microbial diversity compared to those born vaginally, and this consequently resulted in a reduced Th1 response during the initial two years of life, pushing naïve T cells towards a Th2 phenotype, which indicates a greater risk of developing allergies [47]. A study published recently by Martinez II, K. A. *et al* (2017) demonstrated that mice pups delivered by C-section had significantly different gut microbiota compared to pups delivered vaginally [48]. The altered microbiome and reduced taxa of microbiota including *Bacteroides* and *Clostridia*, both known to be important for colonic Treg development, increased the risk of weight gain in pups, demonstrating the importance of a balanced microbiota in normal metabolic development, as well as the immune response [45, 48-50].

The microbiota composition can be affected by antibiotic use for unrelated illnesses, however multiple studies have indicated that almost 50% of antibiotics that are prescribed, have been prescribed unnecessarily which is a serious problem at this current time [51]. Not only does this increase the risk of developing antibiotic resistant bacteria, but it can also cause microbial dysbiosis, which numerous studies have shown to be implicated in several gut pathologies such as Crohn's disease and ulcerative colitis (UC) [52, 53]. The gut microbiota can additionally affect insulin resistance and the risk of diabetes development. It was shown that co-housing obesity-prone C57BL/6 mice from The Jackson Laboratory and obesity-resistant 129S1/SvImJ from the same company suppressed the propensity for obesity development, and the most significant difference between these two strains of mice was their gut microbiota [54]. A greater microbial diversity correlates with a greater population of intestinal Tregs, and this is consequently more protective for the host [50, 55]. Therefore, a greater understanding of the influence of the gut microbiota on

the host immune system will be beneficial to develop more effective, less harsh treatment for intestinal conditions.

Undigested fibrous food within the GI tract provides energy for the microbiota, and can be further broken down into by-products such as short-chain fatty acids (SCFAs) which contain a carbon number between two and six [44, 56]. Fermentation products can lower the intestinal pH and consequently influence the population of gut microbiota which inhabit this environment [57]. Butyrate is one of the most common SCFAs, and alongside certain gut microbiota such as *Clostridia, Bacteroides* and their metabolites, has been shown to induce intestinal pTreg differentiation and number, although they have little effect on nTregs [6, 45, 49]. TCRs expressed by intestinal Tregs have been shown to have increased reactivity to microbial antigens and this is thought to increase the speed of their activation and proliferation [58]. *Clostridia* can colonise the mucosal layer, and exert influence on the intraepithelial cells to produce TGF-β and IDO, both which are important in the differentiation and activation of Tregs respectively [59].

Tregs, predominately within the GALT, are vital to maintain a homeostatic environment largely through secretion of IL-10 [16]. The importance of the gut microbiota in the regulation of the immune system is apparent due to colonic lamina propria Treg numbers and SCFAs being reduced in germ-free (GF) and antibiotic-treated mice compared to WT mice [26, 60]. Similarly, it was demonstrated in an adoptive transfer model that Tregs from WT mice had a better suppressive ability compared to Tregs from GF mice [45]. Neuropilin-1 (NRP-1) and Helios, both markers of nTregs, are both expressed at a low level in $Foxp3^+$ Tregs within this environment, indicating that the intestinal microenvironment favours induction of pTregs [26, 60].

The intestine is a potentially inflammatory environment due to the microbes and food antigens amongst other factors that interact with the mucosal layer. Hence the co-evolution of the intestinal immune system alongside the commensal microbiota is important to prevent excessive inflammation. Within this environment, tTregs, or naïve T cells that migrate to the colonic environment prior to differentiation into pTregs, suppress pro-inflammatory cells via multiple mechanisms, such as antagonising pro-inflammatory effector T cells, through contact-dependent mechanisms such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), and release of soluble cytokines such as IL-10 and TGF-β [3]. Sakaguchi. S, *et al* (1995) demonstrated that

removal of these cells elicited an autoimmune response to certain self-antigens which confirmed their importance in maintaining tolerance [3, 13].

In inflammatory sites such as the intestine, Tregs can co-express effector T cell transcription factors, such as T-bet (Th1), GATA-3 (Th2) or RORɣt (Th17) which can enable a more effective suppressive ability for effector T cells, however they were also considered to be able to 'return' to the inflammatory phenotype depending on the situation [25]. Approximately 40 - 60 % of the colonic p-Treg population co-express RORγt, and the extent of co-expression correlated with microbial diversity [55]. This population was detected only at small levels in other tissues, for example mLNs and spleen [58].

*In vitro* it was demonstrated that naïve $CD4^+$ T cells expressed both RORɣt and Foxp3 when cultured under Th17 inducing conditions, and depending on the balance of pro-inflammatory cytokines and TGF-β levels, this determines whether the cell finally matures into a Treg or effector T cell phenotype [55]. It was thought that this intermediate phenotype could enhance immune cell flexibility for a specific and rapid response to stimuli [61]. However, it was demonstrated *in vivo* that $ROR\gamma t^+Foxp3^+$ Tregs are highly stable within an inflammatory environment, as Treg specific genes; *Foxp3*, *Ctla-4* and *Eos*, were highly demethylated, and are highly suppressive due to increase levels of membrane-bound (m)TGF-β, α4β7, a gut homing integrin and CD62L which is required for immune cell trafficking [55, 61, 62]. Sefik, E. (2015) *et al*, detected IL-17 in $ROR\gamma t^+$ Tregs from the small intestine, however the colonic $ROR\gamma t^+$ Treg population were not found to secrete IL-17, and these Treg populations were negatively affected by broad-spectrum antibiotic treatment [58]. Yang, *et al* (2016) demonstrated that these cells were closer to a Treg phenotype rather than an effector phenotype by isolating $ROR\gamma t^+Foxp3^-$, $ROR\gamma t^+Foxp3^+$ and $ROR\gamma t^-Foxp3^+$ cells from transgenic mice expressing RORc(ɣt)-GFP and Foxp3-IRES-mRFP [62]. Using microarray analysis and next generational sequencing (NGS) it was clearly shown that there was closer clustering of genes between $ROR\gamma t^+Foxp3^+$ Tregs and $Foxp3^+$ Tregs, compared to $ROR\gamma t^+Foxp3^-$ T conventional (Tconv) cells [62].

The importance of these cells was demonstrated by Sefik, E. (2015) *et al*, through a trinitrobenzenesulfonic acid- (TNBS) induced colitis disease model, using WT or Foxp3-Treg specific RORɣt knock-out mice (*Foxp3$^{cre}$.Rorc$^{fl/fl}$*) [58]. There was increased disease activity score and pro-inflammatory cytokine production in the Foxp3-Treg specific RORɣt knock-out mice group compared to WT mice, demonstrating the importance of this subset of Tregs in regulating colonic inflammation [58]. Similarly, they repeated the TNBS disease model using GF mice, monocolonising with various microbes [58]. Disease severity correlated with amount of RORɣt$^{+}$ Tregs, which similarly correlated with microbial colonisation demonstrating the importance of gut microbiota in the development of these cells [58].

Intestinal homeostasis is maintained by DCs that present processed microbial peptides on their surface to induce either effector or regulatory T cells, depending on the signals that were received by the TLRs [63]. Intestinal DCs were thought to have three main subsets; CD103$^{+}$CD11b$^{+}$, CD103$^{+}$CD11b$^{-}$ and CD103$^{-}$CD11b$^{+}$ DCs, which can migrate to lymph nodes and instruct naïve T cells to mature into a distinct subtype [64]. During steady-state conditions, tissue-resident DCs are immature (iDCs), and express low levels of the processing molecule MHC and co-stimulation molecules [65]. Pathogen associated molecular patterns (PAMPs), which are molecules associated with pathogens and are then recognised by the innate immune system, or damage associated molecular patterns (DAMPs), which are endogenous danger signals that signal the body to internal cell death or stress, both of which can be recognised via TLRs, and initiates iDC maturation [65].

TLRs are transmembrane members of the membrane pattern recognition receptor family (PRR), which are able to identify PAMPS and DAMPs. Activation of these receptors initiates various signal transduction pathways, promoting expression of genes involved in the immune response [65]. TLRs detect a diverse range of PAMPs from and trigger an immune response cascade via adapter molecules, such as the myeloid differentiation primary response (MyD)88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) [66]. These adapter molecules activate transcription factors, such as interferon-regulatory factor-3 (IRF-3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) which result in increased expression of cytokines, e.g. tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-ɣ) [66]. This response enables to activation of the adaptive immune response through maturation of DCs and stimulation of other immune cells. Mature

DCs upregulate MHC expression, co-stimulatory molecules for T cell activation, e.g. inducible T-Cell co-stimulator ligand (ICOSL) and C-C chemokine receptor type 7 (CCR7) which enables DC trafficking to local lymph nodes [65].

Migratory DCs ($CD103^+$) express CD103, a TGF-β induced integrin chain. They are found at mucosal surfaces including the intestine and lung. Intestinal DCs are located throughout the villus and lymphoid tissue, vital for sampling and presenting both foreign and self-antigen to T cells [67]. They are thought to be vital in generating Tregs via TGF-β and retinoic acid (RA), a vitamin A metabolite but are also critical for mucosal Th17 cell differentiation [65, 68, 69]. RA can likewise be provided by the intestinal epithelial cells (IECs) which boosts DC expression of retinal dehydrogenases (RALDH) which aids metabolism of dietary vitamin A into RA in a positive feedback loop [65]. $CD103^+$ DCs similarly help inhibit T cell proliferation through production of the enzyme IDO [65, 68, 69]. DCs within the intestinal environment are important for 'sampling' the lumen constituents, they then migrate to mesenteric lymph nodes (mLNs) to activate or suppress immune cells depending on the signals received, upregulating T cell expression of the gut homing molecule CCR9 and αβ-integrin [37, 65]. Intestinal DCs are able to access luminal antigens via extension of their dendrites through the epithelial junctions [16]. At a steady state this can maintain a homeostatic environment via low levels of co-stimulatory molecules, anti-inflammatory cytokine production and low levels of PRR that are used to detect the environmental signals.

Coombes. J. L *et al* (2007), demonstrated that in an *in vitro* cell culture with naïve T cells, only the $CD103^+$ DC subset induced Foxp3 expression [68]. They also investigated the functional properties of $CD103^+$ and $CD103^-$ DCs by culturing them overnight either alone or plus the PAMP lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria, to stimulate TLRs [68]. They previously observed that $CD103^-$ DCs expressed a greater level of TNF-α compared to $CD103^+$ DCs, regardless of whether DCs were stimulated or untreated. TLR stimulation further enhanced $CD103^-$ DCs production of the pro-inflammatory molecules TNF-α and IL-6 which are important for Th17 induction, suggesting that $CD103^-$ DCs are pro-inflammatory, potentially providing protective immunity against the intestinal microbial community, compared to the homeostatic $CD103^+$ DCs [68].

Microbiota, such as *Bacteriocytes* and dietary antigens are also able to induce tolerant DCs [67]. SCFAs act via G-protein coupled receptors (GPCRs) which are located throughout the body, however are expressed greatest on immune cells and intestinal tract epithelial cells [44]. Mice deficient for the butyrate receptor GPR109 had reduced colonic Tregs and were more susceptible to colitis and colon cancer [67]. Intestinal Tregs (and DCs) have the highest expression of the SCFA receptor GPR43 compared to those in the spleen and mLNs demonstrating the importance of microbial products on intestinal immune cell development [70]. SCFAs are dependent on this receptor to act as a HDAC inhibitor and stabilise Foxp3 expression through reduced *Foxp3* gene methylation, thus enhancing suppressive abilities through IL-10 production [44, 71]. Colonic Tregs from a WT mouse had a greater demethylated Foxp3 region and increased stability within the intestinal environment compared to specific pathogen free (SPF) mice [6]. This can influence T cell cytokine profiles, reducing pro-inflammatory cytokine expression and thus preventing excessive inflammation [44, 71]. This has been demonstrated by delivery of SCFAs to GF mice, free of all microorganisms, which promoted an increase in *Foxp3* gene expression [44]. The cooperation between gut microbiota and the immune system is demonstrated by the fact that one current treatment for IBD is immunosuppression, which demonstrates that gut tolerance has broken down, if the inflammation was due to disruptive microbiota, such a suppressed immune response would likely result in overwhelming infection [45].

More recently Nakahashi-Oda, C. *et al* (2016) demonstrated that the subset of $CD103^-$ $CD11b^+F4/80^-$ DCs had a key role in maintaining homeostasis at the rapidly changing interface of the intestinal epithelium, which involved the gut microbiota, while $CD103^+$ DCs acted independently of the microbiome [6, 37]. They demonstrated that $CD11b^+CD103^-$ DCs could produce IFNβ after stimulation by commensal bacteria, and consequently enhanced $Foxp3^+$ Treg proliferation [6]. Type 1 interferons can also help production of IDO, consequently further preventing effector T cell proliferation [65].

BMDCs were generated and cultured with or without caecal content from SPF mice [6]. The BMDCs only produced IFNβ when caecal content was present [6]. Production of IFNβ can be induced by stimulation of TLR4 by LPS and activation of the signal adapter protein TRIF. TRIF forms a complex with either TNF receptor associated factor 6 (TRAF6), to induce activation of NF-κB, or preferentially the serine/threonine protein kinase TANK binding kinase 1 (TBK1) which induces IRF3 to consequently express IFNβ [72, 73]. IFNβ signals via TLR-9 to inhibit intestinal inflammation [74].

Interferons are vital for DC maturation, activation, MHC-II expression and can induce IL-12 to push Th1 cell development [75]. They are a critical intermediary during a response to pathogens and interact with viral, bacterial and antigenic molecules within tissue environments such as the intestine [75, 76]. They were first identified in the 1950's, and currently there are 13 known subtypes of human IFNα, and one version of IFNβ, IFNέ, IFNκ, IFNτ, IFNϭ [76]. Genes are expressed independently of one another, however act in a coordinate fashion, either paracrine or autocrine [76].

Type 1 interferons can inhibit T cell migration from the lymph node which increases their ability to interact with DCs [75]. Multiple groups have shown Type 1 interferons to be involved in improving IBD symptoms. Kole, *et al* (2013) showed the importance of Type 1 interferon signalling in suppressing inflammation in T cell colitis [75]. $CD4^{+}CD45RB^{hi}$ T cells were sorted from WT or $IFNAR^{-/-}$ mice (which lack a major receptor for Type 1 interferons) spleens and transferred into RAG mice. Both groups had similar disease severity due to RAG mice still able to signal via IFNβR [75]. Therefore, RAG and $IFNAR^{-/-}$ mice were crossed and sorted T cells from WT or $IFNAR^{-/-}$ mice were injected i.p to induce T cell colitis [75]. RAG/$IFNAR^{-/-}$ deficient mice injected with WT $CD4^{+}CD45RB^{hi}$ T cells had a greater disease severity than mice injected with T cells from $IFNAR^{-/-}$ mice. Similarly co-transfer of Tregs and T cells into a double knockout host resulted in a loss of Foxp3 expression and these cells gained an effector phenotype, as colonic IFN signalling is important in maintaining Treg homeostasis and suppressive ability, thus effector cell number increases as there is reduced control [75]. Double knockout mice have similar numbers of Tregs compared to WT mice, as multiple signals can induced Tregs as previously discussed, but maintenance of Foxp3 expression and suppressive ability however is greatly affected without type 1 interferon signalling [75].

Type 1 interferons can increase production of anti-inflammatory signals such as IL-10, an IL-1R antagonist from mononuclear phagocytes, and regulatory suppressor of cytokine signalling (SOCS) proteins in mononuclear phagocytes and T cells [75]. Additionally, inhibiting IL-1β production and secretion, preventing Th17 cell differentiation is important within inflammatory settings. This involvement of IFNβ in regulating colonic inflammation was also demonstrated by other groups that saw CpG, mimicking microbiota signalling, induction of IFNβ via $CD11c^{+}$ cells prevented DSS colitis induction [75]. Transcription of *Ifn-β* was dependent of the adapter molecule TRIF [75]. Genome wide association studies (GWAS) have furthermore identified type 1 interferon genes, such as *Ifnar1* and *Stat-3,* downstream of the interferon receptor, as risk loci for IBD [76]. This demonstrates the importance of coordination between immune cells, gut microbiota and subsequent signalling in maintaining homeostasis within the intestine.

### 1.2.2 Disruption of intestinal homeostasis

During normal homeostatic conditions, intestinal immune cells, commensal microbiota and intestinal epithelial cells (IECs) are involved in communication with each other to ensure regulation against excessively reactive immune cells [37]. IECs can sense microbes and influence colonisation through secretion of compounds, and affect the immune system through penetration of the intestinal layer to activate immune cells [37]. Additionally, the mucosal layer, bactericidal compounds, and phagocytic innate immune cells are able to protect against bacterial infiltration through the intestinal wall [16]. However after disruption of the intestinal barrier and infiltration of microbiota, pro-inflammatory Tconv cells are activated by presentation of antigens by APCs in lymph nodes depending on cytokines present [16]. These activated T cells proliferate and migrate into the circulation as memory and Tconv cells which will encounter the antigen at a peripheral site and cause an excessive immune reaction [16].

A breakdown in intestinal homeostasis results in an inflammatory state, and over a long period, this can cause conditions such as Crohn's disease, a form of IBD [16]. This can affect the entire GI tract, however inflammation is mostly focused in the terminal ileum and colon [16]. Ulcerative colitis (UC) is another form of IBD where inflammation is restricted to the colonic mucosal layer [37]. There is no one exact cause of disease development, however it is thought to be a combination of environmental, genetic and host immune factors [37]. GWAS studies show that genes affected in patients with IBD are related to interactions of the gut microbiota with the intestine [16]. Additionally, multiple groups (Gardlick, R. 2015, Leclerc, M. 2011, Pace, N. R. 2010, Engstrand, L. 2010), have shown that gut microbiota is altered in IBD patients compared to healthy controls, which may be a reason for the disruption of the homeostatic environment [77-80]. This demonstrates the importance of the interaction of the gut microbiota with the intestine in maintenance of homeostasis.

Hart *et al* (2005) saw a greater number of activated DCs expressing the marker CD40, and TLRs TLR2 and 4, in IBD patients compared to healthy controls [16, 81]. Additionally, it was shown that Crohn's disease patients had DCs expressing a higher level of the pro-inflammatory cytokines IL-12 and IL-6, pushing naïve T cells towards a Th17 phenotype compared to healthy controls [16, 81]. This demonstrates the importance of understanding the mechanism behind DC tolerance as development of an unnecessary pro-inflammatory phenotype is damaging and could be a potential therapeutic target.

## 1.3 Prostaglandins in Immunity and Inflammation

### 1.3.1 Prostaglandin biosynthesis

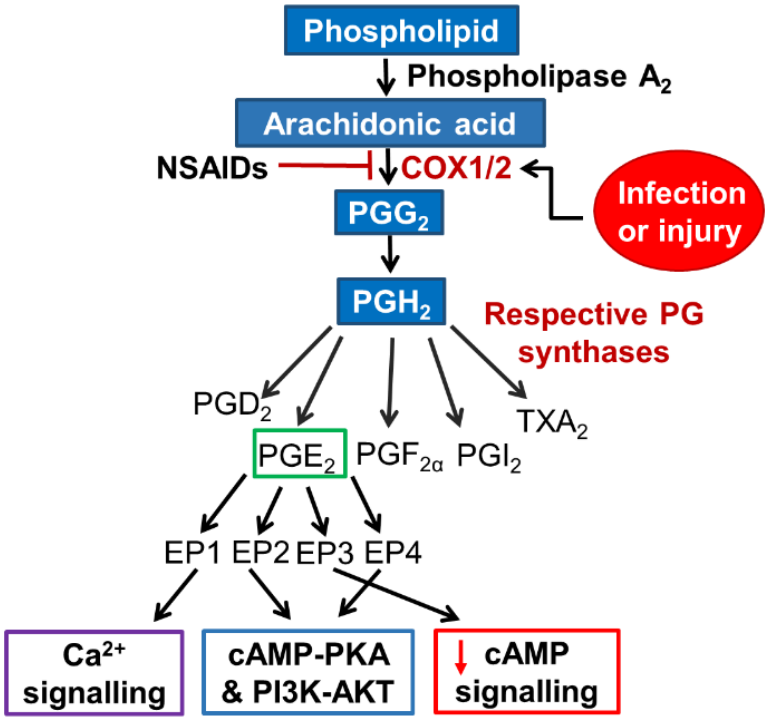


**Figure 1.3: Prostaglandin synthesis**. *The phospholipid membrane is reduced to arachidonic acid by phospholipase $A_2$. This product is then further converted into prostaglandins by the enzymes cyclooxygenase (COX)-1 or COX-2, which are greatly upregulated due to infection or injury. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX activity via blocking the channel where AA binds. The relative prostaglandin synthases which are produced by various cells at site of infection or injury, determine the final subtype of prostaglandin that is formed. Prostaglandin $E_2$ is formed due to the activity of $PGE_2$ synthase (mPGES-1 or -2). This prostaglandin is the focus of this project, and exerts its immunomodulatory activity via four receptors, eicosanoid prostanoid (EP)-1 – 4. Figure adapted from [82].*

Prostaglandins (PGs) are metabolised from phospholipids, by phospholipase A2 (PLA2) into arachidonic acid (AA), a 20-carbon unsaturated fatty acid, then cyclooxygenase (COX)-1 or COX-2 metabolises AA into $PGG_2$ as a response to harmful stimuli (**Fig. 1.3**) [83, 84]. COX-1 is constitutively active in homeostatic conditions and expressed at a low level by most cells. It is required for production of prostaglandins for 'housekeeping' roles such as maintenance of the intestinal epithelial layer [83, 84]. COX-2 is a highly regulated, inducible enzyme after pro-inflammatory stimuli [85].

The structure of COX-1 and COX-2 are very similar, but the active site in COX-2 is larger than in COX-1, a beneficial feature for developing treatments for a specific COX enzyme [86].

Non-steroidal anti-inflammatory drugs (NSAIDs) competitively inhibit both COX enzymes active sites [83, 84]. The downstream product of these enzymes, $PGG_2$ is then further converted into an intermediate compound, $PGH_2$, prior to the interaction with various PG synthases produced at this site of inflammation which determines the final subtype of prostaglandin formed [87, 88]. Prostaglandin synthases consist of oxidoreductase and isomerase which can finally produce the final PG subtype; prostaglandin $D_2$ ($PGD_2$), prostaglandin $E_2$ ($PGE_2$), Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin ($PGI_2$) and thromboxane ($TXA_2$) [83]. The respective synthase expressed by cells at the scene determine the PG outcome, however $PGE_2$ is the most physiological abundant [88].

$PGE_2$ was focused on for this thesis and is converted from $PGH_2$ via membrane-bound PGE synthases (mPGES-1 and mPGES-2) or cytosolic PGE synthase [84]. The majority of $PGE_2$ produced within the body is from APCs and stromal cells, however prostaglandin-endoperoxide synthase (*PTGS2),* also known as cyclooxygenase, has been shown to be increased in T cells after T cell activation [84]. Cytosolic PGE synthase and mPGES-2 are regularly synthesised to maintain a basal levels of $PGE_2$ within the body, however mPGES-1 is upregulated by pro-inflammatory stimuli such as IL-1β, TNFα or bacterial LPS TLR signalling [84]. Prostaglandin synthase enzymes are associated with the Golgi membrane, thus when $PGE_2$ is produced, it is transported across the membrane via an ATP-dependent multidrug resistance protein - 4 or diffusion to act either in an autocrine or paracrine fashion [83]. $PGE_2$ is the most abundant prostaglandin within the body and is expressed by most cells. $PGE_2$ also plays important roles in physiological processes such as maintaining blood pressure as well as influencing the immune system [84]. This molecule exerts its activity via the four rhodopsin-like 7-transmembrane-spanning G-protein coupled receptors EP1 – 4 (**Fig. 1.3**) [83].

EP2 and EP4 both activate the cAMP-PKA pathway via $PGE_2$ stimulation of $G_s$ and adenylyl cyclase, and EP4 can induce PI3K/Akt signalling (**Fig. 1.3**) [83, 89]. However, EP2 is resistant to desensitisation, and has a lower affinity to $PGE_2$ compared to EP4 [85, 87]. The variance in their sensitivities is due to the difference in the length of their C-terminal sequence [84]. EP4 has a longer C-terminus which result in its rapid agonist desensitisation and internalisation. Phosphorylation of residues on the EP4 C terminal chain by PKA and G protein-coupled receptor kinases (GRK) recruits β-arrestin-1 which activates c-Src [90]. This in turn activates the epidermal growth receptor (EGFR) and initiates downstream PI3K and AKT signalling [90]. The isoform EP1 down-regulates cAMP and initiates $Ca^{2+}$ signalling through $G_q$ activation of phosphatidylinositol metabolism (**Fig. 1.3**) [83, 89]. cAMP is a derivative of adenosine triphosphate (ATP), and converted by an isoform of the adenylyl cyclase (of which there are 10) [84]. These vary between cells, and thus have different downstream effects, which gives this molecule a strong mechanism to regulate targeted signalling [84]. Finally, stimulation of the $PGE_2$ receptor EP3 can also inhibit cAMP production and increases intracellular levels of $Ca^{2+}$ after coupling via Gi or G12 [83, 89]. EP3 and EP4 are the most widely receptors through murine tissue and have the greatest affinity for $PGE_2$ [83]. Whereas EP1 is only expressed within certain tissues such as the stomach and lungs, and EP2 is the least abundant [83]. EP receptors are well conserved between mice and humans, therefore *in vivo* mice models can translate into human studies [89].

These receptors are expressed at different levels in multiple cell types and environments, and their various sensitivities and ability to desensitise results in the ability to activate different signal pathways in response to similar signals [87, 91]. Additionally, there are three splice variants of EP3 observed in murine tissue, and eight for humans, adding to the diversity of the $PGE_2$ signal [87].

### 1.3.2 Prostaglandin $E_2$ and the immune response

$PGE_2$ was initially identified as a biologically active molecules in the 1960's, and has been shown to play a role in influencing the role of multiple biological functions, such as blood pressure, intestinal membrane barrier protection, and haematopoiesis, due to the diverse expression of receptors on various cell types [87]. $PGE_2$ can also increase the risk of malignancies due to angiogenesis and suppression of apoptosis [84]. This can be done by downregulation of the Fas ligand (FasL) on immune cells [92].

Under homeostatic conditions, $PGE_2$ is involved in regulation of many biological processes such as; gastrointestinal barrier integrity, fertility, blood pressure, and the immune response [83]. Levels are regulated by the balance of prostaglandin synthase and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) [87]. It is likewise involved in the processes leading to the classic signs of inflammation; rubor, tumour and pain [83, 84]. This information and due to the elevated levels observed at inflammation sites, has resulted in consideration of $PGE_2$ as a pro-inflammatory mediator [93]. Over decades, groups have used COX-2, mPGES-3 and EP deficient mice to confirm the role of $PGE_2$ in inflammation, however this has demonstrated that $PGE_2$ has both a role in the pro- and anti-inflammatory immune response [84].

Previous groups have looked at disease models to determine the role of the COX enzymes and prostanoids within a whole system. The involvement of prostaglandins in inflammation was initially considered in the 1970's as treatment using NSAIDs, non-specific COX inhibitors, prevented acute inflammation development, but this did not provide any information about the involvement of either COX isoform [93]. Through later work using mice deficient for either COX-1 or COX-2, more information has been collected which suggests that the involvement of either COX-1 or COX-2 depends on the tissue affected, how long it has been since damage occurred and what the initial stimuli was [93].

Myers, *et al* (2000) demonstrated the importance of COX-2 in an inflammatory setting through a collagen-induced arthritis (CIA) model [93, 94]. COX-2 deficient mice had reduced inflammation and joint destruction compared to WT mice, whereas COX-1 deficient mice had a similar disease severity compared to WT mice [93, 94]. This may be due to COX-2 being the enzyme upregulated greatest after an inflammatory signal, hence inhibition of this enzyme expression would prevent an inflammatory prostaglandin response, whereas disruption of the housekeeping cyclooxygenase enzyme COX-1 would cause problems with various tissues that are maintained due to low prostaglandin production, such as the intestinal barrier.

However, using a model of dextran sodium sulphate (DSS) colitis, it was demonstrated that COX-2 deficient mice had more severe symptoms compared to both COX-1 deficient and WT mice [93, 95]. This could be caused by the disruption to the intestinal epithelial barrier as a consequence of inhibition of $PGE_2$ production and migration of commensal microbiota enhancing inflammation [93, 95]. This is supported by evidence that IBD patients taking NSAIDs can experience exacerbated intestinal inflammation [93, 96-98]. This demonstrates that unspecific inhibition of COX or more specific inhibition of prostaglandins through the body would have variable results and could have severe side-effects, therefore it is important to understand the role of prostaglandins in more specific environments and their mechanisms of action to be able to regulate this. Especially as inhibiting prostaglandin formation pushes AA towards leukotriene formation which can both increase neutrophil infiltration and enhance inflammation via this molecule [93].

Trebino, C.E. *et al* (2003) used mice deficient for mPGES-1 and induced CIA [83, 99]. They observed that WT mice had worse disease symptoms compared to mPGES-1 deficient mice, while IL-6 and immunoglobulin (Ig)G2a anti-collagen antibody levels were similar between the two groups, demonstrating the importance of $PGE_2$ in inflammation development [83, 99]. Increased inflammation is due to increased permeability of microvesicles within the local environment and thus increased blood flow and pro-inflammatory mediators at the inflamed site [84]. It was later considered that $PGE_2$ also had a role in anti-inflammatory responses due to its ability to induce IL-10 production and supress pro-inflammatory cytokines in some situations to prevent excessive inflammation [87, 93]. Samuelsson, B. *et al* (2007), reviewed papers from multiple groups that used m-PGES-1 knockout mice to demonstrate the importance of $PGE_2$ in regular physiology, and conditions such cancer and arthritis, thus leading to their suggestion of this molecule as a therapeutic target [100].

$PGE_2$'s role is even more complex with immune cells. The increased number of effector T cells detected in the lymph nodes is largely due to $PGE_2$'s ability to induce DC co-stimulatory molecules, to both enhance activation and migration of cells [101]. DCs increased maturation, antigen uptake, and lymph node migration further enhanced this process [84]. Addition of an EP4 antagonist, to prevent $PGE_2$ signalling, suppressed effector T cell expansion in the lymph nodes [102, 103].

Macrophages predominantly produce TXA2 over $PGE_2$ at resting state, however activation results in significant increase in $PGE_2$ production [93]. The difference in prostanoid expression may be down to changes in prostaglandin synthase present, for example after induction of an inflammatory response, IL-1β increases expression of glutathione-dependent PGE-synthase pushing production of $PGE_2$ over TXA2 [93]. $PGE_2$ can activate macrophages via its modulation of cAMP through the key receptors EP2 and EP4, this also increases IFN-ɣ and TNFα production [84]. As well as pro-inflammatory functions, $PGE_2$ can likewise have an anti-inflammatory effect. When macrophages encounter $PGE_2$, this molecule inhibits the effector phenotype and pushes more regulatory functions and cytokines, such as IL-10, after activation of EP2 [87, 88]. Macrophage killing ability is affected by reduction of nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase, and nitric oxide radicals [88].

$PGE_2$ can suppress the effector phenotype of natural killer (NK) cells, a type of lymphocyte in the innate immune system, and production of cytokines such as IFNɣ which is important for protection against both bacterial and viral infections, and cancerous cells [87, 104]. Suppression of $PGE_2$ also prevents activation of macrophages and granulocyte [87, 88]. Thus, in conditions where there is over-production of $PGE_2$ this results in a defective innate immune response [84, 87, 88]. This is largely due to $PGE_2$ - EP2 signalling, as inhibition of the murine EP2 receptor resulted in enhanced bacterial clearance by neutrophils and increased survival of mice infected with *Pseudomonas aeruginosa* [88]. Certain bacteria, such as *Streptococcus pneumoniae* are able to stimulate $PGE_2$ production from neutrophils, acting in a protective manner suppressing the body's first line of defence [88].

$PGE_2$ has an anti-inflammatory activity with the innate immune response, however it has multiple roles in the adaptive immune response.

$PGE_2$ can influence B cells depending on their maturity status. Proliferation of immature B cells is suppressed if $PGE_2$ is present and can induce apoptosis, whereas mature B cells are unaffected by $PGE_2$ presence [92]. However B cell effector function can by influenced by $PGE_2$ induced Ig class switching [92]. In mice, IgG1 and IgE are two classes that can be induced by $PGE_2$, and consequently it can influence asthma and allergy development as these IgG subclasses contribute to a Th2 response [92]. However, it was observed that $PGE_2$ prevented histamine release and mast cell degranulation in sensitised hamsters, which was worsened with indomethacin-treatment [91, 93, 102]. This is likely due to $PGE_2$ pushing naïve T cells towards a Th1 or Th17 phenotype, rather than Th2 [91, 93, 102].

$PGE_2$ is known to negatively affect TGFβ signalling [102]. Our group also showed (unpublished data), that TGFβ-related genes were reduced in microarray expression data from naive murine T cells cultured with $PGE_2$. Increased levels of $PGE_2$ and hepatocyte growth factor (HGF) have been observed to be increased in patients with thoracic malignancies after mesenchymal stem cells (MSCs) transplants. Fibrosis is a problematic side-effect of radiotherapy for these patients, and can result in respiratory failure. MSC transplants resulting in increased $PGE_2$ and HGF reduced fibrosis due to reduction of TGβ-induced collagen and increased cAMP levels inhibiting fibrosis [105].

High levels of $PGE_2$ are observed during chronic inflammation, and can influence the adaptive immune response and development of inflammation by influencing cytokine production by epithelial cells, Tconv cell classes and DC phenotype [93].

### 1.3.3 Prostaglandin $E_2$ and T cells

$PGE_2$ has a multitude of roles when it comes to T cells. The role of $PGE_2$ as a T cell inhibitory molecule through adenyl cyclase and cAMP production, has been investigated since the 1970's, however this occurred at micromolar concentrations [84, 106, 107]. It was later determined that nanomolar concentrations of $PGE_2$ were involved in induction and proliferation of Th1 and Th17 cells, and also T cell proliferation [84].

In the 1970's, it was shown that increased cAMP levels due to $PGE_2$ signalling inhibited T cell activation. Thus, it was thought that $PGE_2$ had an immunosuppressive activity, however it was then realised that the concentration, target and length of signal determined the response of $PGE_2$ signalling [84, 108]. The initial findings reporting $PGE_2$ to be an immunosuppressor were found to be due to inhibition of IL-2 production [84].

In the next decade, more work was carried out determining the role of $PGE_2$ on T cell proliferation and activation which introduced the concept of $PGE_2$ concentrations and the maturation status of target cells potentially affecting the stimulus [84]. Mechanisms for immunosuppression were further investigated and it was observed that inhibition of $Ca^{2+}$, IL-2 and the respective receptor expression was due to $PGE_2$'s activation of the cAMP pathway [84]. This increase in cAMP also reduced internal potassium signalling and thus disrupted signal transduction via the GPCRs and affecting T cell activation and cell proliferation [84].

Betz. M, and Fox. B, S, (1991) then introduced the concept that $PGE_2$ could influence and prevent the differentiation of naïve T cells into Th1 and Th2 cells respectively through suppressing expression of IFNɣ (Th1) and enhancing IL-2, IL-4 and IL-5 (Th2) production [109]. Later studies demonstrated that the cytokine IL-12 and its receptor were also supressed by $PGE_2$, while levels of IL-4, IL-10 and Il-13 were increased [109]. Various mechanisms for prevention of T cell activation by $PGE_2$ have been suggested such as disruption of the protein-kinase C pathways reducing various mitogen-activated protein (MAP) kinase production which normally regulate survival, differentiation and proliferation of T cells [84].

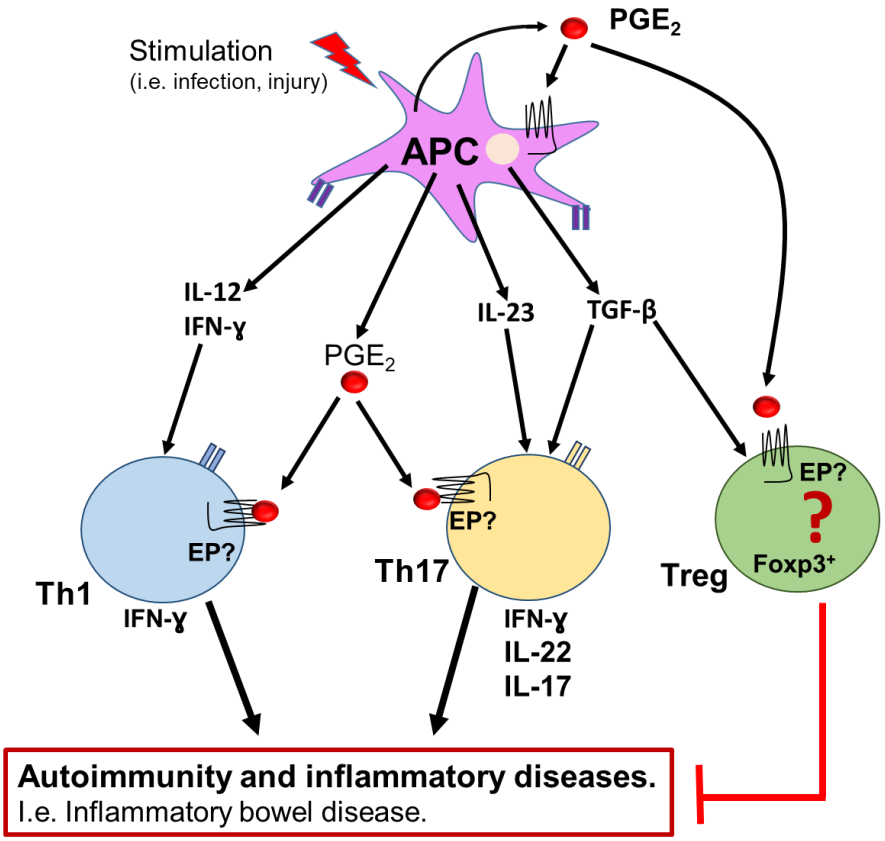


**Figure 1.4: The role of $PGE_2$ in autoimmunity and inflammatory disease development.** *APC stimulation due to infection or injury can significantly upregulate COX expression, enhancing $PGE_2$ production. This can act on Th1 and Th17 EP receptors to enhance differentiation and proliferation of pro-inflammatory Tconv cells. Excessive activation of which can lead to development of autoimmunity and inflammatory conditions such as IBD. However, there is still discussion as to the mechanism by which $PGE_2$ affects Tregs, cells required to suppress pro-inflammatory stimuli.*

Initially the pro-inflammatory role of $PGE_2$ was thought to be dependent on its mechanism of neutrophil recruitment through Tconv cell production of IL-8, however the direct role of $PGE_2$ on Tconv cells was then examined. Yao, C. *et al* (2009) demonstrated that at nanomolar concentrations of $PGE_2$, naïve T cells could be converted to either Th1 or Th17 cells mediated through the EP2 and EP4 receptors and signalling via phosphatidylinositol-3-kinase and PKA (**Fig. 1.4**) [102]. This was further examined using contact hypersensitivity and experimental autoimmune encephalomyelitis (EAE) disease models, where reduced numbers of Th1 and Th17 cells were detected in lymph nodes after administration of an EP4 antagonist [84, 102]. Boniface, K. *et al* (2009) likewise demonstrated the role of $PGE_2$ in human Th17 polarisation via the EP2 and EP4 receptors [103].

$PGE_2$ was shown to upregulate IL-1βR and IL-23R expression via EP2 cAMP-PKA signalling, consequently pushing naïve T cells towards the Th17 phenotype, whereas signalling via EP4 stimulates the PI3K pathway resulting in Th1 cell development [84, 103].

### 1.3.4 Prostaglandin $E_2$ and dendritic cells

$PGE_2$ can also induce DC co-stimulatory molecules, promoting T cell proliferation, additionally it can modify DC cytokine production consequently influencing naïve T cell differentiation [84]. For example, DCs cultured in the presence of $PGE_2$ pushed naïve T cells towards the Th1 phenotype [84]. Whereas LPS stimulated DCs had increased IL-23 production when cultured with $PGE_2$, suggesting that bacterial stimulation and $PGE_2$ presence within the environment pushes naïve T cells towards a Th17 phenotype [84]. $PGE_2$ can similarly induce DC surface co-stimulatory molecules such as CD70, OX40L, promoting T cell proliferation, and modify DC cytokine production which consequently influences naïve T cell differentiation [84]. Co-stimulatory markers were also observed to be upregulated on the surface of T cells, demonstrating a mechanism for sustained cellular interactions and antigenic-specific responses [84]. $PGE_2$ has been shown to suppress differentiation of RA producing DCs, and negatively regulate RALDH production via an inducible cAMP early repressor (ICER)-mediated blockade, the enzyme responsible for RA synthesis [110]. This is potentially as a protective mechanism against infection or damage, demonstrating the influence $PGE_2$ has on immune cell development and differentiation.

### 1.3.5 Prostaglandin $E_2$ and regulatory T cells

$PGE_2$ is known to enhance proliferation of cells, and has been implicated in malignancies and anti-tumour immunity [111]. Baratelli, F. *et al* (2005) demonstrated that $PGE_2$ enhanced the inhibitory ability of Tregs *in vitro* and induced Foxp3 expression in naïve T cells, however this was using tumour supernatants from patients that overexpressed COX-2 or $PGE_2$ at 13 or 26 μM [111]. However it was also thought that $PGE_2$ can negatively affect Tregs due to suppression of immune cell IL-2 production via cAMP signalling, and additionally disruption of the TGF-β signalling pathway, consequently affecting Treg function [87]. Additionally, the recent paper by Wang. X, *et al,* (2017) suggested that cAMP signalling via CREB, which can be activated by $PGE_2$ signalling via EP2 and EP4, aided differentiation of effector Th17 cells, and negatively affected $Foxp3^+$ Treg survival [34]. $PGE_2$ can likewise induce apoptosis in resting mature T cells due to upregulation of c-Myc, consequently modifying the T cell subtypes present within an environment [92]. Most work up till now has been looking at the effect of $PGE_2$ on effector T cells, the work on Tregs is not as clear and has not been demonstrated in an *in vivo* setting. Due to the clear role that $PGE_2$ has enhancing effector T cells differentiation and proliferation, it follows that they have a role on suppression of the cells that would suppress their activation ability (Tregs) due to the importance of regulating immunity through controlling ratios of pro-inflammatory effector T cells (Th17) and suppressor cells (Tregs).

### 1.3.6 Prostaglandin $E_2$ and the intestine

$PGE_2$ has both pro- and anti-inflammatory roles in the intestine. It is 'cytoprotective' and can prevent intestinal damage and ulceration, through reducing gastric acid secretion and enhancing secretion of protective mucous [86]. It can signal via the key receptor EP4, to activate the innate lymphoid cell - 3 (ILC-3) - IL-22 pathway, which can protect against gut epithelial barrier injury via enhancing mucin and tight junction gene expression (**Fig. 1.5**) [112]. $PGE_2$ also enhanced ILC-3 IL-23-driven IL-22 production, which is an epithelial regenerative factor, and colony stimulating factor-2 (CSF-2) to push DC RA production to induce protective Tregs [39, 112]. Thus, due to $PGE_2$'s alternate protective roles within the intestine and more widely through the body, such as protection of the intestinal barrier and suppression of pro-inflammatory neutrophil responses, NSAID long-term use is not a suitable treatment [112]. NSAIDs can severely exacerbate symptoms, especially as increased leukotriene production due to arachidonic acid after inhibition of COX-1 and COX-2 can increase neutrophil infiltration [113].

Although $PGE_2$ has been shown to have a protective role in the intestine, upregulation of the molecule has also been observed during intestinal inflammation, as well as in various cancers [113]. Induction of inflammation within the intestine, enhances production of COX-2 and consequently secretion of $PGE_2$ by intestinal tissue [113].

$PGE_2$ can increase vascular permeability and prevent electrolyte absorption, resulting in some of the most common IBD symptoms, enhanced bowel movements and diarrhoea [113]. After acting in either an autocrine or paracrine fashion, $PGE_2$ is metabolised by 15-PGDH in the colon into 15-keto-$PGE_2$, then in the liver and kidney it is further broken down into 13,14-dihydro-15-keta $PGE_2$ by Δ13-reductase [113]. Following β-oxidation and ὼ-oxidation, PGE-MUM is formed and excreted in the urine, therefore increased levels of $PGE_2$ correlate with increased PGE-MUM [113].

For inflammatory bowel conditions; such as UC, it is important to monitor the mucosal healing of patients, as long-term damage can result in greater negative side-effects such as bacterial infiltration and sepsis [113]. However currently the methods to do this are very invasive, colonoscopy or biopsies and add further discomfort to patients [113]. Arai, Y *et al* (2014) looked at measuring the $PGE_2$ metabolite, prostaglandin E-major urinary metabolite (PGE-MUM) excreted in urine from patients with UC, as a sign of mucosal healing and compared to C-reactive protein (CRP) levels [113, 114]. This was considered due to the link between $PGE_2$ levels and colonic inflammation, additionally increased levels were found to correlate with poor disease activity score [113, 114]. This marker was used because another key inflammatory marker, CRP, may not be increased even in patients with active colitis, so was not a reliable marker for this condition [113, 114]. This demonstrates the importance of $PGE_2$ in intestinal inflammation, and treatment involving suppression of $PGE_2$ is currently being considered to allow tissue recovery, suppression of disease relapse, and prevention of colon cancer development [113]. All risks associated with increased intestinal inflammation.

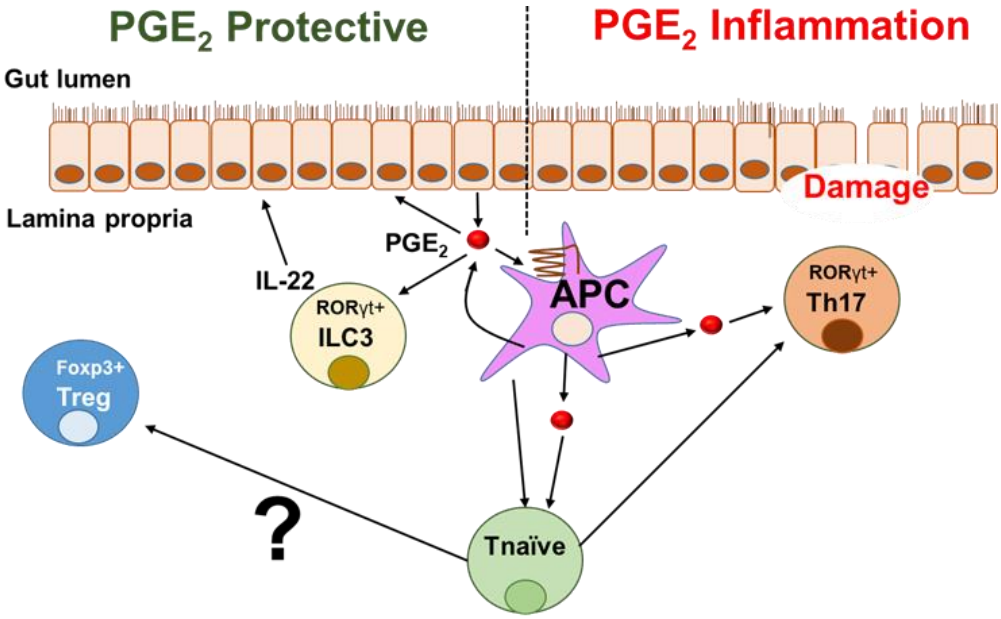


**Figure 1.5: The role of $PGE_2$ in the intestine.** *$PGE_2$ has both a pro- and anti-inflammatory response within the intestinal environment. $PGE_2$ can be produced by various cells within the intestine, and can induce Th17 proliferation and activity, excessive activation of which can result in intestinal damage and inflammation due to potential bacterial infiltration. Whereas $PGE_2$ can also act on ILC3s and enhance IL-22 production resulting in protection and maintenance of the intestinal barrier and the mucosal surface thus protecting the intestine from foreign pathogens and excessive inflammation. However, the role that endogenous $PGE_2$ plays on intestinal Tregs is not known. Figure adapted from [112, 115].*

The multitude of roles that $PGE_2$ has within the immune system indicates that simple inhibition of prostaglandin production is not a long-term viable solution to reduce inflammation without causing severely negative side-effects. Therefore, research into how to manipulate this environment in a more specific and localised fashion, whilst enhancing anti-inflammatory Treg to suppress inflammation would benefit the 300,000 people in the UK who have IBD (numbers taken from the UK Inflammatory Bowel Disease Audit by the Royal College of Physicians in 2014) [116].

### 1.3.7 Treatments targeting prostaglandin $E_2$

$PGE_2$ has an important role in the immune system as previously discussed, therefore controlling this to influence disease development is important, and treatment to reduce inflammation due to this molecule has been used since the late 1800s. Acetylsalicylic acid (aspirin), originating from salicylate in willow bark, was initially introduced in 1897 and patented by Bayer in 1899, and since then multiple similar products have also been developed [117]. The NSAIDs initially developed were 'broad-spectrum', in that they inhibited both COX-1 and COX-2, however the issue with this is that COX-1 is vital for homeostatic processes, such as maintaining gut barrier integrity therefore inhibition of both enzymes can have some significant side-effects due to damage of the intestine [117]. However epidemiological studies showed a reduced risk of neuroinflammatory disease such as Alzheimer's disease or Parkinson's disease after long-term NSAID treatment, but the significant side-effect included intestinal bleeding [118, 119]. The issue is with the pharmacology of the NSAIDs; indomethacin, ibuprofen and aspirin are more active against COX-1 than COX-2, which affects the housekeeping roles of prostaglandins and as such cause greater gastrointestinal damage, but has less of an impact on the prostaglandin production within the inflammatory sites with upregulated levels of COX-2 [86].

Therefore, more 'specific' NSAIDs were developed to inhibit COX-2 [117]. Celecoxib is an example of a specific COX-2 inhibitor, which prevents the risk of intestinal ulcers and bleeding, however they had significant side-effects and resulted in increased pro-thrombotic risk and greater incidences of heart attacks and strokes [117].

Intestinal inflammation due to conditions such as colitis would benefit from inflammatory cell suppression, however it is difficult to do this efficiently without exacerbating the condition. Therefore, this strengthens the importance of understanding the mechanisms of $PGE_2$ on immune cells to harness this.

### 1.3.8 Genetic evidence for the role of $PGE_2$ in inflammatory diseases

Robb, T. C. *et al* (2017) observed that increased $PGE_2$ production by dermis cells enhanced IL-17 and IL-22 production by Th17 and other activated T cells [120]. These cytokines are vital components of allergic contact dermatitis (ACD) and atopic dermatitis (AD) pathogenesis [120]. Patients are found to have increased levels of IL-22 in the serum and inflamed skin compared to healthy controls which stimulated epidermal hyperplasia [120]. Laouini, *et al* (2005) observed that inhibition of $PGE_2$ increased Th2 and eosinophil cell infiltration, resulting in worse disease severity in ovalbumin-sensitised mice [121]. Whereas other groups, including Robb, *et al* (2017) observed that $PGE_2$ pushes Th2 conversion and B cell infiltration, and via the key EP receptors, EP2 and EP4 also increased Th17 and Th22 pro-inflammatory cytokine IL-17 and IL-22 production [120].

The intestine is a highly variable environment with the immune system and gut microbiota in close contact, thus maintaining homeostasis is key to preventing unnecessary colonic inflammation. Several groups have observed a link between long-term aspirin use and a reduced risk of colorectal cancer [122-126]. In 2009, the UK government published a Drug Safety Update permitting the prescription of low dose aspirin for patients who already have vascular disease [124, 127]. Following this, in 2015, the United States Preventative Services Task Force included colorectal cancer prevention in the recommendation for low dose aspirin, in addition to its protective role against cardiovascular disease [128]. Aberrant expression of the COX enzymes, notably COX-2 which is greatly upregulated in an inflammatory environment, increases $PGE_2$ expression within both pre-malignant and malignant colorectal tumours [84, 129]. Fink, S. P. *et al* (2014) discovered that aspirins protection against colorectal cancer was even more effective in patients with elevated levels of mucosal hydroxyprostaglandin dehydrogenase-15 (15-PGDH), compared to those with low levels of this enzyme, suggesting that inhibition of prostaglandin synthesis enzymes, and robust degradation of $PGE_2$ are required to reduce the risk of colorectal cancer [125].

COX enzymes and their consequent prostaglandin products were first implicated in colorectal tumorigenesis back in 1983 when Gardner's syndrome patients, who develop multiple colon polyps, had a reduced adenoma burden after NSAID treatment [123, 129]. Excessive intestinal inflammation can result in cancer development, as well as IBD conditions. IBD patients also have an increased risk of colon cancer, it accounts for approximately 15 % of all deaths for IBD patients, this further cements the need for understanding the effects of $PGE_2$ on cells within the intestine [130, 131].

The gene *PTGER4,* encoding EP4, has been suggested to be a susceptibility locus influencing a variety of human inflammatory and autoimmune diseases including multiple sclerosis (MS) and IBD [102, 132-136]. Matesanz, *et al* (2012) did a meta-analysis of MS GWAS data, and detected the *PTGER4* single nucleotide polymorphisms (SNP) rs4613763 to be suggestive of MS risk, and Esaki, et al (2010) demonstrated that EP4 deficient mice were protected from EAE development [133, 137]. Risk of allergy development can also be increased by SNPs at the *PTGER4* locus rs7720838 [138]. Mutations in *PTGER4* have similarly been shown to be involved in autoimmune conditions such as Ankylosing Spondylitis (AS) due to a polymorphism at SNP rs10440635 [139, 140]. This condition depends on the Th1 or Th17 response, however this suggests $PGE_2$ can also affect the Th2 response, and the SNPs identified within this study can help identify elements that influence the balance between Th phenotypes [138]. Fungus or bacteria can activate the caspase-associated recruitment domain 9 (CARD9) signalling pathway after activation of dectin-1 and dectin-2, and consequently enhance expression of $PGE_2$ [139, 140]. Signalling via EP4 triggers Th17 induction through production of the cytokines IL-23 and IL-17 which enhances risk of development of both Crohn's and AS development [139, 140]. Additionally, $PGE_2$ can aid bone remodelling which is a feature of AS [139, 140]. Increased expression of this gene was detected in synovial biopsies from AS patients compared to controls [140].

Further studies saw that mutations at the *PTGER4* locus rs7720838 and rs4495224 correlated with PTGER4 expression, and this was shown to be an important susceptible gene for risk of IBD [141, 142]. This risk was due to increased ability for the transcription factors NFκB and X-box binding protein (XBP1) to bind, thus enhancing expression of the *PTGER4* gene [141, 142].

These GWAS studies provide evidence that the $PGE_2$-EP4 signalling pathway is involved in immune system development. Thus, this further cements the importance of understanding the mechanism of $PGE_2$ actions *in vivo*, and a benefit of looking at EP receptors is that they are well conserved between mice and humans, so using mouse models can allow translational work into human studies [89].

## 1.4 Rationale, Hypothesis and Aims for this study

Emerging evidence has shown that $PGE_2$ critically regulates inflammatory Th1 / Th17 cells, but how $PGE_2$ effects Tregs is not fully understood. Given the essential roles for both Th1/ Th17/ Treg cells, and the $PGE_2$ signalling pathway in regulation of chronic inflammatory responses, it is imperative to study the precise mechanisms for how $PGE_2$ affects Tregs, especially tissue-resident Tregs in steady state and under inflammatory conditions.

### 1.4.1 Hypothesis

The hypothesis for this PhD is that ‘‘$PGE_2$ signalling inhibits Foxp3 expression and consequently affects Treg induction and function, which facilitates intestinal inflammation.’’

### 1.4.2 Aims

The aims of the thesis were:

- To identify the role of $PGE_2$ on *in vitro* Treg induction.
- To determine the mechanism for how $PGE_2$ regulates intestinal resident Tregs.
- To study how $PGE_2$ regulates inflammatory diseases by modulating Tregs.

# 2 Materials and methods

## 2.1 Mice

Wild-type C57BL/6 mice were obtained from Charles River or Harlan UK and housed at the University of Edinburgh animal facility at the Little France campus. The EP4 knockout mice were generated by crossing C57BL/6 lox-flanked *Ptger4* (EP4-floxed) mice with Lck-Cre mice (on C57BL/6 background) to generate mice with T cell-specific EP4 deletion [143]. Foxp3-YFP, IFNRA$^{-/-}$ and MyD88/TRIF DKO genetically modified mice (bred to congenicity on a C57BL/6J Ola Hsd background) were bred and maintained under specific pathogen-free conditions in accredited animal facilities at the University of Edinburgh [144-147]. Age- and sex-matched mice, older than seven weeks old, were used for experiments. Mouse tissue extraction was performed in accordance to U.K. Home Office legislation. Mice were analysed individually and none were excluded from the analysis, with the exception of exclusions due to technical errors in preparation of intestinal lamina propria lymphocytes (LPLs). All experiments were conducted under project licences issued in in accordance with the UK Scientific Procedures Act of 1986 and had local ethical approval.

## 2.2 General Reagents

### 2.2.1 Media

#### 2.2.1.1 Wash media

During preparation of bone marrow dendritic cells (BMDCs), femurs removed from C57BL/6 mice were transferred into cryovials containing RPMI-1640 medium (Sigma Aldrich) to soften attached tissue. Contents of the marrow were flushed with wash medium (RPMI-1640) prior to re-suspending in cell culture medium (described below) for plating.

#### 2.2.1.2 Culture media

For all *in vitro* human and murine cell culture, cells and compounds were re-suspended in medium consisting of RPMI-1640 medium supplemented with 50 µM β-mercaptoethanol (β-ME) (Gibco Life Technologies), antibiotics (penicillin and streptomycin, 100 U/ml, Gibco Life Technologies), 2 mM L-glutamine (PAA Laboratories Ltd) and filtered 10 % heat-inactivated foetal calf serum (FCS, Gibco Life Technologies). All subsequent references to FCS refers to FCS heat inactivated at 56 $^{o}$C for forty-five minutes in a water bath to inactivate complement.

**Table 1***: List of compounds used during experimental procedures, assay concentrations and supplier. Final assay concentration stated in experimental figure legend.*

| Compound | Final assay concentration | Supplier |
|---|---|---|
| **Prostaglandin $E_2$** | 10 -1000 nM | Cayman Chemicals |
| **17-phenyl trinor $PGE_2$** | 1000 nM | Cayman Chemicals |
| **Butaprost (EP2 selective agonist)** | 1000 nM | Cayman Chemicals |
| **L-902,688 (EP4 selective agonist)** | 1000 nM | Cayman Chemicals |
| **PF-04418948 (EP2 selective antagonist)** | 10,000 nM | Cayman Chemicals |
| **L-161,982 (EP4 selective antagonist)** | 10,000 nM | Cayman Chemicals |
| **db-cAMP** | 25,00 - 100,000 nM | Sigma / Calbiochem |
| **H-89** | 10,000 nM | Sigma / Calbiochem |
| **LY-294002** | 10,000 nM | Sigma / Calbiochem |
| **Recombinant human TGF-beta (rhTGFβ)** | 0.1 - 10 ng/ml | Biolegend |
| **Indomethacin** | 1– 5 mg/kg body weight/day | Sigma |
| **Collagenase Type IV** | 1.25 mg/ml | Gibco Life Technologies |
| **DNase-I** | 30 ug/ml | Roche |
| **Recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF)** | 20 ng/ml | Miltenyi Biotec |
| **Phorbol 12-myristate 13-acetate (PMA)** | 0.1 µg/ml | Sigma |
| **Ionomycin** | 1 µg/ml | Invivogen |
| **Golgi plug** | 0.2 µg/ml | BD Biosciences |

## 2.3 Immune Cell Isolation

### 2.3.1 Isolation of cells from the spleen

Spleens were removed from the mouse and macerated between microscope slides to form a single cell suspension in ice cold phosphate-buffered saline (PBS) (in-house)/ 2 % FCS. Cells were filtered through a nylon mesh then spun at 400 x G-force (g) in the Rotina 420R centrifuge (Hettich Zentrifuge) for 10 minutes, supernatant removed then Ammonium-Chloride-Potassium (ACK) lysing buffer (ammonium chloride 150 mM, potassium hydrogen carbonate 10 mM, Ethylenediaminetetraacetic acid (EDTA) Fisher Scientific 0.1 mM) was added for four minutes at room temperature (RT) to lyse red blood cells. PBS/2 % FCS was added to stop the lysis reaction, and cells spun again before being re-suspended in PBS/2 % FCS prior to staining. Total cells were counted using a haemocytometer.

All tissue culture was performed in class II safety cabinets using relevant plastics produced by Costar (Corning-Costar, Schiphol-Rijk, Netherlands).

### 2.3.2 Isolation of cells from the mesenteric lymph nodes (mLNs)

After removal from the mouse, the mLNs were mashed through a 40 µM sterile nylon mesh cell strainer (Fisher Scientific) to form a single cell suspension in ice cold PBS/2 % FCS buffer. Cells were spun at 400 x g for 10 minutes, and then re-suspended in PBS/2 % FCS prior to further use.

### 2.3.3 Isolation of cells from the colon

Intestinal lamina propria cells were isolated as previously described by Bain *et al* (2012) [148]. Colons were extracted and their contents removed by first using forceps to gently apply pressure, then cutting the colons open and swirling them in a petri-dish containing ice cold PBS to remove excess faecal matter. Tissues were cut into small pieces into 50 ml centrifuge tubes containing cold 2 % FCS, 1X Hanks' Balanced Salt Solution (minus calcium chloride, minus magnesium chloride) (HBSS) (Gibco Life Technologies), and shaken vigorously in parallel for 30 seconds before poured through sieves.

The tissue was transferred into tubes containing cold 2 mM EDTA, HBSS; a chelating solution to aid dissociation and disaggregation of the tissues, then incubated for 15 minutes at 37 $^{o}$C while shaking at 200 RPM in the Orbital Incubator (Stuart Scientific SI 50). The tissue was poured back through the sieve, then washed after being transferred into a tube containing pre-warmed HBSS and shaken as above. In subsequent stages, all medium was pre-warmed.

Tissues were poured through the sieve then transferred back into 2 mM EDTA, HBSS, and incubated for 30 minutes as before in the Orbital Incubator. The chelating solution was poured through the sieve, then tissues washed with HBSS before being transferred to gentleMACS tubes (Miltenyi Biotec) containing digestion solution consisting of; collagenase-4 (Gibco, Life Technologies) and DNase-1 (Roche) in cell culture medium.

The tissue was further cut into smaller pieces and incubated at 37 $^{o}$C for 30 minutes while shaking at 200 RPM. To further dissociate colonic tissue, the gentleMACS tubes were attached to the gentleMACS dissociator and the murine intestine programme was run. Subsequently, tubes were placed on ice and the colon single cell suspension was poured through 40 µM strainers and rinsed with PBS/2 % FCS. Cells were spun down at 400 x g for 10 minutes, and ACK buffer was added at room temperature. After 3 - 5 minutes, ice cold PBS/2 % FCS was added, cells spun down then re-suspended in PBS/2 % FCS prior to further use.

### 2.3.4 Isolation of cells from the small intestine

To maximise cell viability during tissue processing, the small intestine was stored in ice-cold culture medium. Any excess fat or visible Peyer's patches were gently removed from the small intestine before it was cut open and swirled in a petri-dish containing ice-cold PBS to remove excess faecal matter, then transferred into culture medium. The small intestine was cut into small segments and added to 50 ml centrifuge tubes containing extraction medium (culture medium and 0.5 M EDTA). Tubes were incubated for 15 minutes at 37 $^{o}$C while shaking at 200. After the incubation, contents were drained through the sieves then transferred back into tubes containing pre-warmed culture medium, which were then shaken vigorously in parallel for 30 seconds. Contents were poured through the sieves as before, then transferred into gentleMACS tubes containing digestion solution made up as described in **section 2.3.3**.

The tissue was chopped into smaller pieces, then incubated in the shaker at 37ºC for 15 minutes. The gentleMACS tubes were attached to the gentleMACS dissociator and the murine intestine programme was run. Subsequently, tubes were placed on ice and colons mashed through 40 µM strainers using a 2 ml syringe plunger (BD Plastipak), rinsed with PBS/2 % FCS. Cells were spun down at 400 x g for 10 minutes, and ACK buffer was added at room temperature. After 3 - 5 minutes, ice cold PBS/2 % FCS was added, cells spun down then re-suspended in PBS/2 % FCS. Protocol was adapted by Aleksandra Prochera [149].

### 2.3.5 Isolation of cells from the bone marrow

To prepare BMDCs, femurs were removed from C57BL/6 mice and transferred into tubes containing wash medium to loosen muscle tissue. The muscle tissue was dissected from the bone which was then briefly placed into 70 % ethanol to sterilise the exterior. The ends of the femur were trimmed using sterile, sharp scissors to expose the interior marrow shaft, and contents of the marrow were flushed with wash medium using a 1 ml insulin syringe with a 29 G × ½ needle. The cell suspension was transferred through a 40 µm cell strainer into a 50 ml falcon tube, then centrifuged at 300 x g for five minutes and supernatant discarded. The cell pellet was re-suspended in ACK lysis buffer for 15 seconds then 10 % complete medium was added and centrifuged at 300 x g for five minutes. The cells were washed twice then counted using a haemocytometer prior to re-suspending the bone marrow cells at 1 x $10^7$ cells/ml in culture medium with 20 ng/ml recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF).

Bone marrow cells were gently added to a petri-dish containing culture medium and 20 ng/ml GM-CSF, for a final cell concentration of 4 x $10^5$ cells/ml. The petri-dish was gently swirled to ensure that solutions were mixed, then cells were incubated at 37 °C, 5 % $CO_2$ and 95 % humidity in a $CO_2$ incubator for three days.

On day three, medium was replenished by gently adding warm 10 % complete medium with 20 ng/ml GM-CSF to each petri-dish. To minimise disturbance of cells, medium was pipetted down the edge of the petri-dish. The petri-dish was gently swirled to ensure solutions were mixed then incubated for a further three days. This medium replenishment was repeated on day six, and on day nine, bone marrow derived cells (BMDCs) were harvested by collecting the non-adherent cells after gently swirling the plate [150].

The cell suspension was centrifuged at 300 x g for five minutes then re-suspended in PBS/2 % FCS prior to further use.

### 2.3.6 Preparation of caecum content

After mice treated with vehicle, indomethacin, or indomethacin plus an EP4 agonist were culled, caecum content was immediately extracted, transferred into an eppendorf tube on dry ice and stored at $^{-}$80 $^{o}$C until required. Caecum matter was diluted in sterile PBS (100 mg/ml), vortexed vigorously, then spun at 10.0 x 1000 for 2 minutes (Biofuge Benchtop Centrifuge). The solution was strained through a 0.22 µM Millex GP filter unit (Fisher Scientific) and frozen at $^{-}$20 $^{o}$C for use in *in vitro* cell cultures.

## 2.4 Cell Purification

**Table 2:** *A list of antibodies used for flow cytometry and cell sorting staining. All items were purchased from eBioscience, Biolegend and Cell Signalling.*

| Antibody | Conjugate | Clone |
|---|---|---|
| **CD4** | PerCP-Cy5.5 | RM4-5 |
| | PE | GK1.5 |
| **CD25** | APC | PC61.1 |
| | BV605 | PC61 |
| | FITC | AF488 |
| **Foxp3** | FITC | FJK-16s |
| | PE | FJK-16s |
| **eFluor670** | 670 (FITC) | N/A |
| **eFluor780** | 780 | N/A |
| **CD45** | APC-Cy7 | 30-F11 |
| | Efluor450 | 30-F11 |
| **IFNγ** | FITC | RA3-6B2 |
| **IL-17a** | PerCP-Cy5.5 | ReBio17B7 |
| **CD11b** | BV605 | M1/70 |
| | PE | M1/70 |
| | FITC | M1/70 |
| **CD11c** | FITC | N418 |
| | APC | N418 |
| **Ly6G** | APC | 1A8-Ly6g |
| **CD103** | FITC | 2E7 |
| **MHCII** | AF700 | M5/114-15.2 |
| **RORɣt** | PerCp-eF710 | B2D |
| **Ki67** | Bv605 | 16A8 |
| **pSMAD2/3** | PE | 072-670 |
| **CD3** | AF700 | eBio500A2 |
| | PE | 145-2C.1 |
| | APC | 145-2C11 |
| **pSTAT-1** | APC | |

### 2.4.1 Purification of $CD4^+$ splenocytes

Spleens removed from Foxp3-YFP mice were processed as described in **section 2.3.1**. CD4 microbeads (1:9) (Miltenyi Biotec) were added to the single cell suspension and incubated at 4 ºC for 30 minutes. $CD4^+$ cells were positively selected using the AutoMACS pro (Miltenyi Biotec) then re-suspended in PBS/2 % FCS before a sample was taken to be counted using the haemocytometer. The $CD4^+$ isolated cell population was adjusted to 1 x $10^6$ cells/ml in cell culture medium for initial *in vitro* experiments.

In later experiments (as stated in the results section), $CD4^+$ T cells were stained for 30 minutes at 4 ºC with antibodies for CD25 (fluorophores listed in **table 2**). After washes, cells were re-suspended in PBS/2 % FCS for sorting by the FACS Fusion (QMRI or SCRM) into live nTregs ($CD4^+$, $CD25^+$, Foxp3-$YFP^+$) and live naïve T cells ($CD4^+$, $CD25^-$, Foxp3-$YFP^-$). Cells were collected in tubes containing PBS/10 % FCS.

### 2.4.2 Purification of dendritic and mononuclear phagocyte cells

The mLN, BMDCs and colon single cell suspensions were stained for 30 minutes at 4ºC with antibodies for CD45, CD3/B220. CD11c, MHCII, CD11b and CD103 (fluorophores listed in **table 2**). After two washes, cells were re-suspended in PBS/2 % FCS for sorting by the FACS Fusion (QMRI or SCRM) into $CD45^+$, $CD3/B220^-$, $CD11c^+$, $MHCII^+$, $CD11b^+$, and $CD103^+$ or $CD103^-$ DCs and mononuclear phagocytes (MNPs). Cells were collected in tubes containing PBS/10 % FCS.

## 2.5 *In vitro* Cell Culture

### 2.5.1 iTreg and nTreg cell culture

Anti-mouse CD3 (αCD3) and anti-mouse CD28 (αCD28) (both purchased from eBioscience) were diluted in PBS to 10 and 5 ng/ml respectively, and added to wells of a 96 well cell culture flat bottom plate (3595 Costar Corning). The plate was incubated at 37 ºC for two hours, then washed three times with ice cold PBS before cells were plated. The sorted naïve T cell ($CD4^+CD25^-$Foxp3-$YFP^-$) or nTreg ($CD4^+CD25^+$Foxp3-$YFP^+$) cell population was adjusted to 1 x $10^5$ cells/ml in cell culture medium. TGF-β was added to the required wells on day 0, then cells incubated at 37 °C, 5 % $CO_2$ and 95 % humidity in a $CO_2$ incubator. $PGE_2$ and other compounds were added as specified in **Table 1** on day 1. Cells were usually harvested on day 3 (unless stated otherwise), to stain for Treg markers for flow cytometry analysis.

*In vitro* cell cultures were performed in triplicates and repeated three or more times as stated in the relevant results sections.

### 2.5.2 Dendritic cell and naïve T cell co-culture

Sorted mLN derived $CD103^+$ and $CD103^-$ DCs were adjusted to 1 x $10^4$ cells/well, and sorted splenic naïve T cells were adjusted to 0.5 x $10^4$ cells/well on day 0, and plated in a 96 well cell culture round bottom plate (3595 Costar Corning). Soluble αCD3, TGF-β and IL-2 (details in **table 1**) were then added. Cells were incubated for 24 hours at 37 °C, 5 % $CO_2$ and 95 % humidity in a $CO_2$ incubator before medium or caecal content addition. Cells were incubated for a further 48 hours at 37 $^oC$ before harvesting to stain for Treg markers for flow cytometry analysis.

### 2.5.3 BMDC differentiation

BMDCs sorted for $CD45^+$, CD3/ $B220^-$, $CD11c^+$, $MHCII^+$ and $CD103^-$ cells were re-suspended at 1 x $10^6$ cells/ml in culture medium, then sterile filtered caecal content, previously prepared as stated in section 2.3.5, was diluted 40X in culture medium prior to addition to the cells. BMDCs were stimulated with the caecum contents for either 2.5 or 6 hours at 37 $^oC$ with 5 % $CO_2$, then washed twice with PBS/2 % FCS and re-suspended in buffer RLT and β-ME, and stored at $^-80$ $^oC$ for future PCR work. The timings for this experiment were chosen due to previous experiments demonstrated in a paper by Nakahashi-Oda. C, et al [6].

## 2.6 Cell Staining and Flow Cytometry

### 2.6.1 Processed tissue stain

Tissues were processed to a single cell suspension as described in **sections 2.3.1 - 2.3.4**, then plated in a 96 well cell culture round bottom plate (3595 Costar Corning). Total cells were counted using a NucleoCassette *(Chemometec)* counter. Plates were spun at 400 x g for 5 minutes, then cells stained with the live/dead marker Fixable Viability Dye eFluor™ 780. Cells were incubated for 30 minutes at 4 $^oC$, then washed twice with PBS/2 % FCS. Cells were subsequently stained for either Treg or DC surface markers (antibody details in Table 2, markers described in results section) and incubated for 30 minutes at 4$^oC$ prior to washes with PBS/2 % FCS. Cells were spun down, re-suspended in fixation/permeabilization buffer (eBioscience) and incubated at 4 $^oC$ for two hours. Cells were washed with permeabilization buffer (eBioscience) prior to Treg or DC internal marker staining.

Antibodies were diluted in permeabilization buffer (antibody details in **Table 2**, markers described in results section), and incubated for an hour at 4 ºC. Cells were washed twice then re-suspended in permeabilization buffer, and transferred into FACS tubes before samples were run on the BD LSR 5 laser Fortessa (BD bioscience).

To boost cytokine expression prior to staining for inflammatory cytokines such as IL-17 or IFNɣ, phorbol 12-myristate 13-acetate (PMA), ionomycin and a Golgi Plug inhibitor were added to the single cell suspension re-suspended in culture medium, then incubated at 37 ºC, 5 % $CO_2$ for four hours. Cells were re-suspended and washed in PBS/2 % FCS before being stained with the live/dead marker diluted in PBS-only. Cells were incubated for 30 minutes at 4ºC, then washed twice with PBS/2 % FCS. Cells were fixed with fixation/permeabilization buffer overnight, then washed with permeabilization buffer before staining for cytokines and other cellular markers (antibody details in Table 2, markers stated in results section) for one hour. Cells were washed twice then re-suspended in permeabilization buffer, transferred into FACS tubes and run on the BD LSR 5 laser Fortessa (BD bioscience).

### 2.6.2 Cell culture stain

On day 4 of the *in vitro* cell cultures, medium was aspirated from the wells and prestoblue cell viability reagent (Invitrogen) was added (diluted 1:10 in pre-warmed cell culture medium). Cells were incubated at 37 ºC for 30 minutes before absorbance (570 nm) and fluorescence (528/20 nm, 590/20 nm) were detected by the computer programme Gen5 1.11 on the Synergy HT plate reader (Biotech). Cells were re-suspended in PBS, transferred to a 96 round-bottom well plate and washed three times at 530 x g for five minutes. The live/dead marker was added to wells, and cells were incubated at 4 ºC for 30 minutes before being washed with PBS. Surface staining antibodies were diluted in PBS and added to the cells preceding incubation at 4 ºC for 30 minutes. After cells were washed and supernatant was removed, fixation/permeabilization buffer was added and cells incubated at 4 ºC for two hours. Cells were subsequently washed with permeabilization buffer, then stained for internal markers for one hour. Cells were washed twice then re-suspended in permeabilization buffer and transferred into FACS tubes before samples were run on the BD LSR 5 laser Fortessa.

## 2.7 Detection of Gene Expression

### 2.7.1 RNA purification and cDNA reverse transcription

Colon tissues from mice treated with either vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) were processed as discussed in **section 2.3.3**. Colonic MNPs were sorted for $CD45^+$ $CD3/B220^-$ $CD11c^+$ $MHCII^+$ and $CD103^-$ or $CD103^+$, then collected in tubes containing PBS/10 % FCS. Cells were washed with PBS/2 % FCS then re-suspended in buffer RLT (QIAGEN) and beta-mercaptoethanol (β-ME) (Sigma-Aldrich), and stored at $^-80^oC$ for future work.

nTreg and iTreg cells that had been cultured with either vehicle (0.04 % dimethyl sulfoxide (DMSO)) or $PGE_2$ (100 nM) were also treated like this to detect Foxp3 gene expression.

BMDCs that had been incubated with medium, or sterilely filtered vehicle, indomethacin or indomethacin plus an EP4 agonist caecum content, for either 2.5 or 6 hours were washed then lysed in buffer RLT and β-ME. These samples were used to determine whether gene expression was altered between different culture conditions.

A small section of colon tissue was excised after culling of treated mice prior to tissue processing and stored at $^-80$ $^oC$ for subsequent PCR use to detect total gene expression.

Ribonucleic acid (RNA) purification from lysed cell samples or tissue samples was performed using the Rneasy Mini Kit (Qiagen).

#### 2.7.1.1 Cell samples

Lysed cell samples stored at $^-80$ $^oC$ were thawed before addition of 70 % ethanol at a 1:1 ratio. Samples were rapidly vortexed then transferred into the Rneasy mini-spin columns. Tubes were spun at 10,000 RPM for 30 seconds then supernatant removed. Buffer RW1 was added before spinning again prior to buffer RPE addition. After spinning at 10,000 revolutions per minute (RPM) for 3 seconds, supernatant was discarded. Buffer RPE was added and then tubes spun for 2 minutes at 10,000 RPM. After discarding the flow-through, tubes were centrifuged at 10,000 RPM for 1 minute to dry the membrane. The Rneasy mini-spin column was transferred into a new collection tube, before RNase-free water was added directly to the spin column membrane. Tubes were spun at 10,000 RPM for 1 minute to elute RNA.

#### 2.7.1.2 Tissue samples

A small section of frozen colon tissue was transferred into a labelled falcon tube and buffer RLT and β-ME was added in addition to two metal beads. The tubes were placed in a shaker rack and shaken for two minutes at 30 hertz. Tubes were then spun at 10,000 x g for 3 minutes and the processed tissue solution was transferred into a clean tube. The ensuing polymerase chain reaction (PCR) protocol was similar to the above steps for cell preparation (**section 2.7.1.1**), starting with the addition of 70 % ethanol at a 1:1 ratio, until the addition of RNase-free water directly to the spin column membrane. Tubes were spun at 8,000 for 1 minute to elute RNA, then the NanoDrop was used to calculate the volume of sample required for the RNA concentration to be 1 µg in 14 µl, diluted in RNase-free water.

#### 2.7.1.3 cDNA reverse transcription

Both the cell and tissue sample RNA was processed following the subsequent steps. Complementary deoxyribonucleic acid (cDNA) was synthesised by reverse transcription using the High-capacity cDNA Reverse Transcription Kits (ABI). The PCR reaction mixture consisted of: 10 X RT buffer, 25 X dNTP mix, 10 X RT random primers, Multiscribe$^{TM}$ Reverse Transcriptase and nuclease-free $H_2O$. The PCR reaction mixture was added in a 1:1 ratio to the eluted RNA, then samples were run on the PCR machine (G-Storm) using the cDNA RT programme. Cycling conditions: lid heated to 100 $^{o}$C; 25 $^{o}$C for 30 minutes; 37 $^{o}$C for two hours; 85 $^{o}$C for five minutes; then samples held at 4 $^{o}$C.

The cDNA from cell samples was diluted 1:6 with deionised water, whereas cDNA from tissue samples was diluted 1:10. Samples were stored at $^{-}$20 $^{o}$C from this point.

### 2.7.2 Real-time PCR

The cDNA reaction mix (GoTaq qPCR Master Mix, Promega) consisted of $dH_2O$, SYBR premix (2x), Dye II (50 x), and primers for gene of interest (10 µM), made up in that order. The following primers were used (**Table 3**).

***Table 3*****:** *List of primers and their forward and reverse sequence.*

| | **Forward** | **Reverse** |
|---|---|---|
| **Foxp3** | 5'-GCCCAGACCCCTGTGCT-3' | 5'-CCGGGAGCACACTGCCC-3' |
| **IL-10** | 5'-TGAGGCGCTGTCGTCATCGATTTCTCCC-3' | 5'-ACCTGCTCCACTGCCTTGCT-3' |
| **IFNα** | 5'-GGACTTTGGATTCCCGCAGGAGAAG -3' | 5'-GCTGCATCAGACAGCCTTGCAGGTC-3' |
| **IFNβ** | 5'-AACCTCACCTACAGGGCGGACTTCA-3' | 5'-TCCCACGTCAATCTTTCCTCTTGCTTT-3' |
| **IFNγ** | 5′-TGAACGCTACACACTGCATCTTGG-3′ | 5′-CGACTCCTTTTCCGCTTCCTGAG-3′ |
| **ISG15** | 5'-TGACTGTGAGAGCAAGCAGC-3' | 5'-CCCCAGCATCTTCACCTTTA-3' |
| **IRF7** | 5'-CCCCATCTTCGACTTCAGAG-3' | 5'-AAGGAAGCACTCGATGTCGT-3' |
| **GAPDH** | 5'-ACAGTCCATGCCATCACTGCC-3' | 5'-GCCTGCTTCACCACCTTCTTG-3' |
| **18S** | 5'- AAGTCCCTGCCCTTTGTACACA-3' | 5'-GATCCGAGGGCCTCACTAAAC-3' |

Tubes were vortexed and kept on ice while preparing the Applied Biosystems 7900HT microAMP FAST 96-well reaction plate. The cDNA reaction mix was added in a 1:4 ratio to the cDNA, and plate covered with a microAMP RT-PCR cover. The plate was spun for 30 seconds at 530 x g then run on the PCR machine (Applied Biosystems) using the SDS 2 programme. Cycling conditions: 95 $^{o}$C for two minutes (stage 1); 95 $^{o}$C for one second and 60 $^{o}$C for 20 seconds, 40 cycles (stage 2); 95 $^{o}$C for 15 seconds, 60 $^{o}$C for 15 seconds and 95 $^{o}$C for 15 seconds (dissociation step). Expression was normalised to mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S and presented as relative expression to control group by the $2^{-\Delta\Delta Ct}$ method.

## 2.8 *In Vivo* Management of $PGE_2$ Signals and Depletion of Gut Microbiota

### 2.8.1 Inhibition of endogenous prostaglandin expression

To determine the effects of endogenous $PGE_2$ in vivo, wild type (WT) C57Bl/6 or various transgenically-modified mice were treated with either vehicle (0.5 % ethanol), the non-steroidal anti-inflammatory drug (NSAID) indomethacin (5 mg/kg/day) to block endogenous $PGE_2$ production, or indomethacin plus an EP4 agonist (10 µg/day, i.p) to specifically activate a key $PGE_2$ receptor for five to seven days. On day seven mice were culled and tissues removed for processing. Both vehicle and indomethacin were administered in the drinking water, whereas the EP4 agonist was injected intraperitoneally (i.p) daily.

To see whether a lower dose of the NSAID indomethacin still influenced immune cell differentiation, a long-term treatment was performed. WT C57Bl/6 mice were treated with either vehicle (0.25 % ethanol) or indomethacin (2 or 1 mg/kg/day) for two or four weeks respectively before being culled and tissues removed for processing.

The genetically modified MyD88/TRIF double knock-out (DKO) and interferon receptor deficient ($IFNAR^{-/-}$) mice were treated with the same concentration of vehicle and indomethacin as the seven day treated WT mice, albeit only for five days before being culled due to concerns over bacterial translocation across the intestinal barrier potentially resulting in infection.

Organs were collected, and processed as mentioned in **sections 2.3.1 – 2.3.5**. Caecum matter was extracted from the mice treated with the vehicle, indomethacin, or indomethacin plus an EP4 agonist and stored as stated in **section 2.3.6.**

### 2.8.2 Depletion of gut microbiota

Wild-type C57Bl/6 mice were treated with vehicle (0.5 % ethanol), or broad-spectrum antibiotics to deplete the gut microbiota (**Table 4**) in their drinking water a week prior to addition of vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) in the drinking water for a further week. On day 14, mice were culled and organs extracted for processing as mentioned in **sections 2.3.1 – 2.3.5.** The antibiotic cocktail has been previously utilised in multiple papers to deplete gut microbiota [112, 151].

**Table 4:** *Antibiotic cocktail plus sucralose (4 mg/ml) used for in vivo work to deplete gut microbiota.*

| **Antibiotics** | **Concentration (mg/ml)** |
|---|---|
| Ampicillin | 0.5 |
| Gentamycin | 0.5 |
| Metronidazole | 0.5 |
| Neomycin | 0.5 |
| Vancomycin | 0.5 |

## 2.9 Disease Models

### 2.9.1 Dextran sodium sulfate (DSS) colitis

To determine the importance of $PGE_2$ signalling in disease progression, $LCK^{Cre}$ and $EP4^{fl/fl}$ (control) or $LCK^{Cre}EP4^{fl/fl}$ (T cell specific EP4 knockout) mice were given 2 % Dextran Sulfate Sodium Salt – colitis grade (DSS: MP Biomedicals) in drinking water on day 0, and disease severity was scored daily. After eight days, mice were culled and colons extracted and treated as stated in **section 2.3.3.**

Another method to prevent $PGE_2$ signalling is to use the NSAID indomethacin to inhibit prostaglandin production. Therefore, WT C57BL/6 mice were treated with either water only, 2 % DSS or 2 % DSS plus indomethacin for eight days and scored daily. On day 8, mice were culled and processed for flow cytometry. Duffin. R, *et al* (2016) have previously described severity scoring [112].

In brief, the scoring chart works out the cumulative score between stool appearance: 0, normal; 1, soft; 2, soft and presence of dry blood; 3, loose with fresh blood; 4, sticks to fur. Detection of blood was scored on the following scale: 0, normal; 1, dark blood; 2, fresh blood in stool; 3, blood around anus; 4, fresh blood. Weight loss was scored on percentage loss from initial weight: 0, 0 – 4 %; 1, 5 – 9; 2. 10 – 14; 3, 15-19; 4, 20 – 24; 5, 25. General appearance was scored by these various parameters: 0, normal; 1, piloerect; 2, piloerect and lethargic; 3, Ataxic, motionless and sunken eyes. A validated clinical disease activity index ranging from 0 to 17 was calculated using the following parameters: Stool consistency, presence of faecal blood, general appearance, and changes in body weight. If mice achieved a score of 11 or above, or their weight loss exceeded 25 % of their initial weight, then mice were culled.

### 2.9.2 T cell colitis

T cell colitis was induced in $RAG^{-/-}$ mice. $RAG^{-/-}$ mice do not have any mature B or T lymphocytes, therefore any damage that is induced in these mice after T cell transfer is solely due to the donor cells initiation. Spleens were extracted from either WT or EP4 knockout mice and processed as stated in **section 2.3.1**. Naïve T cells ($CD4^{+}CD25^{-}$) were sorted on the FACS Aria Fusion (BD Biosciences) and washed five times with PBS-only to ensure that all serum was removed. These cells were used to determine whether preventing $PGE_2$ signalling in naïve T cells had a protective effect when injected into RAG mice during induction of T cell colitis. Cells were re-suspended at 2.5 x $10^6$ cells/ml and 0.5 x $10^6$ cells/ml were injected by i.p per mouse. Mice were scored weekly until clinical symptoms were observed then they were scored three times weekly. Mice were culled after six weeks, and colons prepared for histology, PCR and processed as stated in **section 2.3.3** and stained for both Treg markers and cytokines to be detected using flow cytometry.

Mice were clinically assessed using the following criteria: Weight loss was scored on percentage loss from initial weight: 0, 0 – 4 %; 1, 5 – 9; 2. 10 – 14; 3, 15-19; 4, 20 – 24; 5, > 25. General appearance was scored by these various parameters: 0, normal; 1, piloerect or lethargic; 2, piloerect and lethargic; 3, piloerect, lethargic and sickly appearance; 4, ataxic, motionless and sunken eyes. The ocular score was determined by the following symptoms: 0, normal; 1, puffy eyes; 2, puffy eyes, scratching, and skin inflammation. The scores are combined to provide a total colitis score for each mouse (ranging between 0 – 11). Mice were culled if their weight loss is greater than 25 %, total score reaches nine or above for two consecutive days, general appearance is scored 4, or the ocular score 2 is noted for two consecutive scoring days.

### 2.9.3 Faecal transplant and DSS colitis

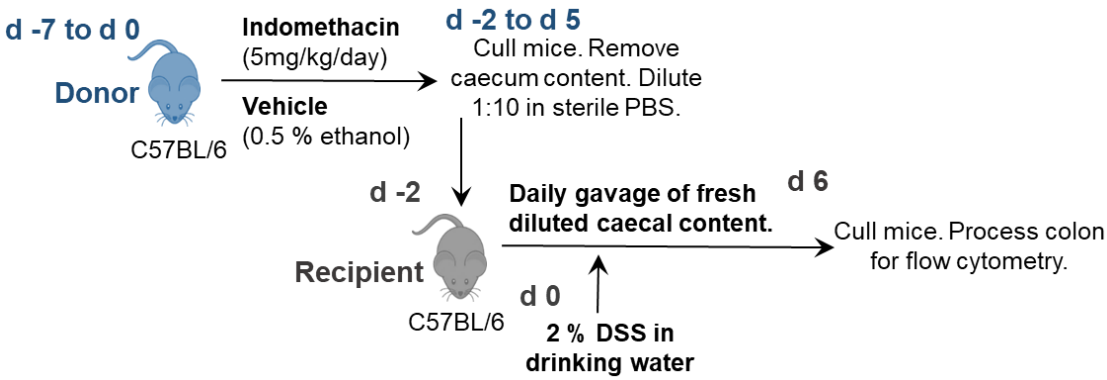


**Figure 2.1: Experimental timeline for faecal transplant disease model.** *Eight groups of donor mice were treated with either indomethacin (5 mg/kg/day) or vehicle (0.5 % ethanol) for five days starting in a sequential manner. Mice were culled and caecum content removed under sterile conditions. Caecum content was diluted 1:10 with sterile PBS, then gavaged daily into recipient mice. Recipient mice were gavaged with fresh caecum content from day -2 until day 6, and treated with 2 % DSS colitis in drinking water from day 0 until day 6.*

To determine the effect of gut microbiota on immune cells and consequent disease development, C57BL/6 mice were treated with either indomethacin (5 mg/kg/day) or vehicle (0.5 % ethanol) for five days (donor mice) (**Fig. 2.1**). On day 5, donor mice from either the vehicle or indomethacin treated group were culled, and colon and caecum content removed. This content was weighed and diluted 1:10 in cold PBS, vortexed then gravity settled for two minutes prior to administration by gavage to another group of C57BL/6 mice (recipient mice) for eight days. On day 0 of the experimental protocol, DSS colitis was induced in the two groups of recipient mice (gavaged with caecum content from either indomethacin or vehicle treated mice) by 2 % DSS in water, and disease severity was scored daily (scoring system same as stated above in **section 2.9.1**). Recipient mice were also weighed on day -2 in case faecal matter transfer influenced weight. On day 6, mice were culled, colons were processed as stated in **section 2.3.3** and stained for both Treg markers and cytokines to be detected using flow cytometry.

## 2.10 Statistical Analysis

All data were expressed as mean ± SEM. Statistical significance between two groups was examined by the Mann-Whitney test, while the Kruskal-Wallis test with Dunn's Multiple Comparison test for post-hoc comparison. was used to evaluate multiple groups. Statistical work was performed using Prism 6 software (GraphPad) and a p value of less than 0.05 was considered significant.

# 3 The Role of $PGE_2$ on $CD4^+$ T cells *In Vitro*

## 3.1 Introduction

Prostaglandins (PGs) are lipid mediators derived from arachidonic acid (AA), a product released from the phospholipid membrane by phospholipase $A_2$ in response to harmful stimuli like infection and injury [87, 88]. The prostaglandin synthase produced by cells migrating to the site of damage result in the specific PG production.

$PGE_2$ exerts its actions by binding to its receptors termed eicosanoid prostanoid (EP)-1, EP2, EP3 and EP4. While EP2 and EP4 activates cAMP-PKA and PI3K/Akt pathways, EP3 down-regulates cAMP signalling and EP1 initiates $Ca^{2+}$ signalling. These receptors are expressed at different levels in various cells types and environments, and their differing sensitivities and abilities for desensitisation results in the ability to activate assorted signal pathways in response to similar signals [88].

EP2 and EP4 are similar in that they signal via the cAMP-PKA and PI3K-AKT pathways, and are both principally responsible for the anti-inflammatory and regulatory properties of $PGE_2$ [88, 152].

For decades it was believed that $PGE_2$ suppresses the T cell responses due to downregulation of TCR signalling, as well as reducing IL-2 and IFNγ responsiveness and production, pushing naïve T cells away from a Th1 phenotype [109]. Sharma. S *et al* (2005), in agreement with other groups, saw increased levels of COX-2 and $PGE_2$ in non-small cell lung cancer (NSCLC) and saw in mice that this correlated with increased Tregs [153-155]. Similarly, Treg numbers were reduced by genetically inhibiting COX-2 [87]. Baratelli. F, et al (2005) observed an increase in Foxp3 expression in human Tregs and naïve after a 24 hour culture with $PGE_2$ [111]. Supporting Sharma's work, they also saw an increase in Tregs after culturing naïve T cells with supernatant from COX-2 overexpressing tumour supernatant [111].

However other groups have seen alternative results from $PGE_2$. Ricciotti. E, *et al* (2011) saw that the rheumatoid synovium in murine collagen induced arthritis (CIA) produced high levels of $PGE_2$ which correlated was implicated in the production of inflammatory cytokines IL-1 and IL-6 [83]. Mice deficient in EP4, preventing $PGE_2$ signalling, had reduced levels of these pro-inflammatory cytokines and fewer CIA symptoms [83]. This pro-inflammatory role of $PGE_2$ was also supported by Sakata. D, *et al* (2010) who demonstrated that EAE severity was greatly reduced in both wild-type and EP4 knock-out mice after use of an EP4 antagonist [156]. There was also reduced accumulation of Th1 and Th17 cells in lymph nodes, demonstrating the importance of $PGE_2$ signalling in inflammatory responses [156]. Additionally, previous work has shown how $PGE_2$, via the receptors EP2 and EP4, increases the differentiation of Th1 cells and enhances IL-23 production by APCs to enable Th17 expansion [91, 102]. Therefore, $PGE_2$ has been shown to effect both effector T cells during inflammation, and although some groups have shown it to boost Treg numbers, these conflicting results demonstrate that the function and role of $PGE_2$ is still not fully understood.

Further evidence for $PGE_2$'s role in inflammation is that current treatment for autoimmune and chronic inflammatory conditions include non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit COX-1 and COX-2 activity, and subsequent prostanoid synthesis [157]. Considering that Tregs suppress T cell-mediated inflammation, this raised the question whether $PGE_2$ also influenced Treg cells. Additionally, the different responses that $PGE_2$ can exert on cell types is due to the diversity and expression of the various EP receptors, likewise the varied sensitivities and ability for desensitisation of the receptors [87, 88]. It was then questioned how $PGE_2$ affected Treg development in an *in vitro* cell culture.

This subsequent chapter follows on from Dr Chengcan Yao's preliminary work, which saw a reduction in Treg genes following a microarray of both human and murine cells cultured with vehicle or $PGE_2$.

## 3.2 Methodology

To determine the role of $PGE_2$ on T cell Foxp3 expression, spleens were removed from C57BL/6 mice then macerated between microscope slides to form a single cell suspension in ice cold PBS. Cells were filtered and red blood cells (RBCs) lysed by ACK lysing buffer, then incubated for 30 minutes with CD4 microbeads prior to positive selection using the AutoMACS pro. In initial experiments, whole $CD4^+$ T cell populations were used for *in vitro* culture (stated in specific results section). In later experiments, isolated $CD4^+$ T cells from Foxp3-YFP mice were stained with antibodies for CD25 (APC), and sorted on the FACS Fusion into live nTregs ($CD4^+$, $CD25^+$, Foxp3-$YFP^+$) and live naïve T cells ($CD4^+$, $CD25^-$, Foxp3-$YFP^-$).

Wells of a 96 well cell culture flat bottom plate were coated with αCD3 and αCD28, and incubated at 37 $^oC$ for two hours prior to washing with PBS and cell addition at 1 x $10^5$ cells/ml diluted in cell culture medium. Details of compounds added are described in more detail in the sections below but in brief, TGF-β was added to cells on day 0, then following 24 hours incubating in at 37 °C, 5 % $CO_2$ and 95 % humidity in a $CO_2$ incubator, $PGE_2$ or the relevant compounds were added. On day 3 cells were harvested and stained for Treg markers using flow cytometry or PCR.

### 3.2.1 Aims of chapter

- To determine how $PGE_2$ regulates TGF-β-induced Treg development *in vitro*.
- To define the mechanism by which $PGE_2$ suppresses the TGF-β response during Treg development.

## 3.3 Results

### 3.3.1 $PGE_2$ negatively impacts the TGF-β response

The role of $PGE_2$ on regulatory T cells has been widely discussed, however there is no clear consensus on the effect on this compound on Tregs. Some groups have found that overexpression of cyclooxygenase 2 which correlates with $PGE_2$ levels resulted in increased Foxp3 and Treg levels within the tumour microenvironment [158]. Whereas other groups have shown it to negatively affect Foxp3 expression, Chen, H. *et al* (2009), observed that mRNA levels of Foxp3 were increased in TGF-β only treated murine naïve T cells, however this was suppressed by $PGE_2$ presence [159]. More recently, Yao. C, et al (2009, 2013) clearly observed both *in vitro* and *in vivo* that this lipid molecule enhanced differentiation and proliferation of Th1 and Th17 cells, hence further evidence it would be expected to reduce levels of antithetical Tregs [91, 102].

Preliminary work suggested that addition of $PGE_2$ suppressed Foxp3 expression when naïve T cells were cultured with TGF-β. Therefore, to see whether $PGE_2$ still affected the TGFβ response at increased concentration, $CD4^+$ T cells were sorted from C57BL/6 mouse splenocytes using the AutoMACs, then stimulated with αCD3/αCD28 and TGF-β (0 – 10 ng/ml) on day 0. $PGE_2$ at 100 nM) and vehicle (0.04 % DMSO) were added to the cell culture on day 0. Cells were harvested and Foxp3 expression detected by flow cytometry on day 3 (**Fig. 3.1a**). The gating strategy for analysis of $CD25^+Foxp3^+$ Tregs in live $CD4^+$ T cells is demonstrated in **figure 3.1b**.

TGF-β dose-dependently induced Foxp3 expression in $CD4^+$ T cells, which was markedly suppressed by $PGE_2$ at 100 nm, despite the higher TGFβ concentration (**Fig. 3.1c**). This dose of $PGE_2$ also affected viable $CD4^+$ cells (**Fig. 3.1d**). However, the inhibitory effect on Foxp3 was observed most clearly at the highest levels of TGFβ stimulation (5, 10 ng/ml) (**Fig. 3.1c, e**). There was 55 % Foxp3 expression in cells cultured with TGF-β at 10 ng/ml and vehicle-only, whereas this expression was only 16 % and for cells cultured with 100 μM $PGE_2$ respectably (**Fig. 3.1d**). Additionally, there was a steep increase in both percentage and number of Foxp3 expressing cells between those cultured with TGF-β at 1 ng/ml and 5 ng/ml, whereas this was not observed with cells additionally treated with $PGE_2$ (**Fig. 3.1c, d**). These data suggest that $PGE_2$ affects TGF-β's induction of Foxp3 expression. (**Fig. 3.1d, e**). Therefore, the following experiments were conducted using 10 ng/ml TGF-β and 100 nM $PGE_2$ [160].

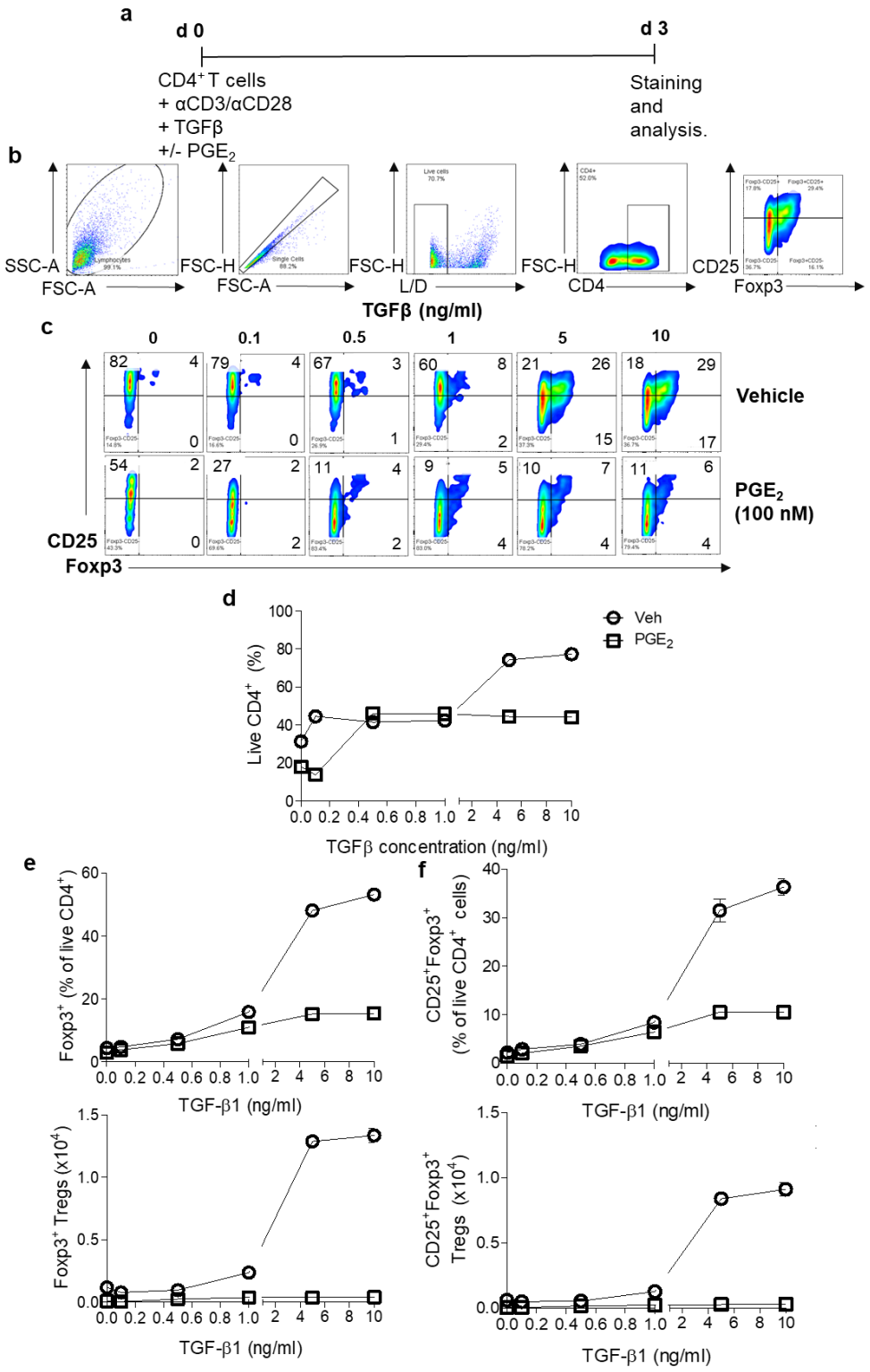


**Figure 3.1: Effect of $PGE_2$ on the TGF-β response.** *(a) Experimental timeline for in vitro cell culture. Freshly isolated CD4⁺ T cells were stimulated with αCD3/αCD28 and indicated concentrations of TGF-β plus or minus $PGE_2$ at 100 nM. On d 3, cells were harvested and intracellularly stained with anti-mouse CD25 and Foxp3. (b) Flow cytometry dot plot illustrates*

*the gating strategy for Foxp3 and CD25 expression in live $CD4^+$ T cells. (c) Flow cytometry dot plots of CD25 and Foxp3 expression in live $CD4^+$ T cells cultured with various concentrations of TGF-β in the absence or presence of $PGE_2$ for three days. (d) Percentage of live $CD4^+$ cells. (e) Percentages and number of $Foxp3^+$ or $CD25^+Foxp3^+$ cells within total live $CD4^+$ T cells under indicated conditions (mean ± standard error of the mean (SEM) of triplicates). Data represents one experiment. Representative of 3 independent experiments.*

### 3.3.2 $PGE_2$ suppression of Foxp3 expression is independent of its inhibitory effects on T cell activation, proliferation and viability

It was observed in **figure 3.1** that there were fewer viable cells in the $PGE_2$-treated group compared to the vehicle-treated group, indicating that the suppression of Foxp3 expression may be secondary to its negative effect on cell viability. This is partially due to $PGE_2$ being known to suppress TCR activation [87].

To test whether delayed $PGE_2$ addition would improve cell viability, but also still prevent Foxp3 induction, $CD4^+$ T cells sorted from splenocytes using the AutoMACs, were stimulated with αCD3/αCD28 and TGF-β on day 0, and $PGE_2$ (0 – 1000 nM) was added either on day 0 at the beginning of T cell activation, or day 1, 24 hours after TCR stimulation. Cells were harvested and Treg markers were detected by flow cytometry on day 3 (**Fig. 3.2a**).

Postponed addition of $PGE_2$ partially rescued T cell viability as well as T cell activation, assessed by expression of CD25 (**Fig. 3.2b, c, d**). However, $PGE_2$ still dose-dependently suppressed Foxp3 expression similarly despite its addition time (**Fig. 3.2b, c, d**). This suggests that postponed addition of $PGE_2$ does not rescue Foxp3 expression, even though there is increased cell viability and activation.

To see whether gene expression was also affected, naïve T cells were treated with either vehicle or $PGE_2$ added on day 1. Cells were harvested and processed for PCR. It was observed that $PGE_2$ similarly prevented Foxp3 gene expression in naïve sorted $CD4^+CD25^-$Foxp3-$YFP^-$ T cells compared to in vehicle-treated cells (**Fig. 3.2e**).

Taken together, these results indicate that $PGE_2$ suppression of Foxp3 induction is unlikely, if any, to have resulted from its inhibitory effects on T cell activation, or viability.

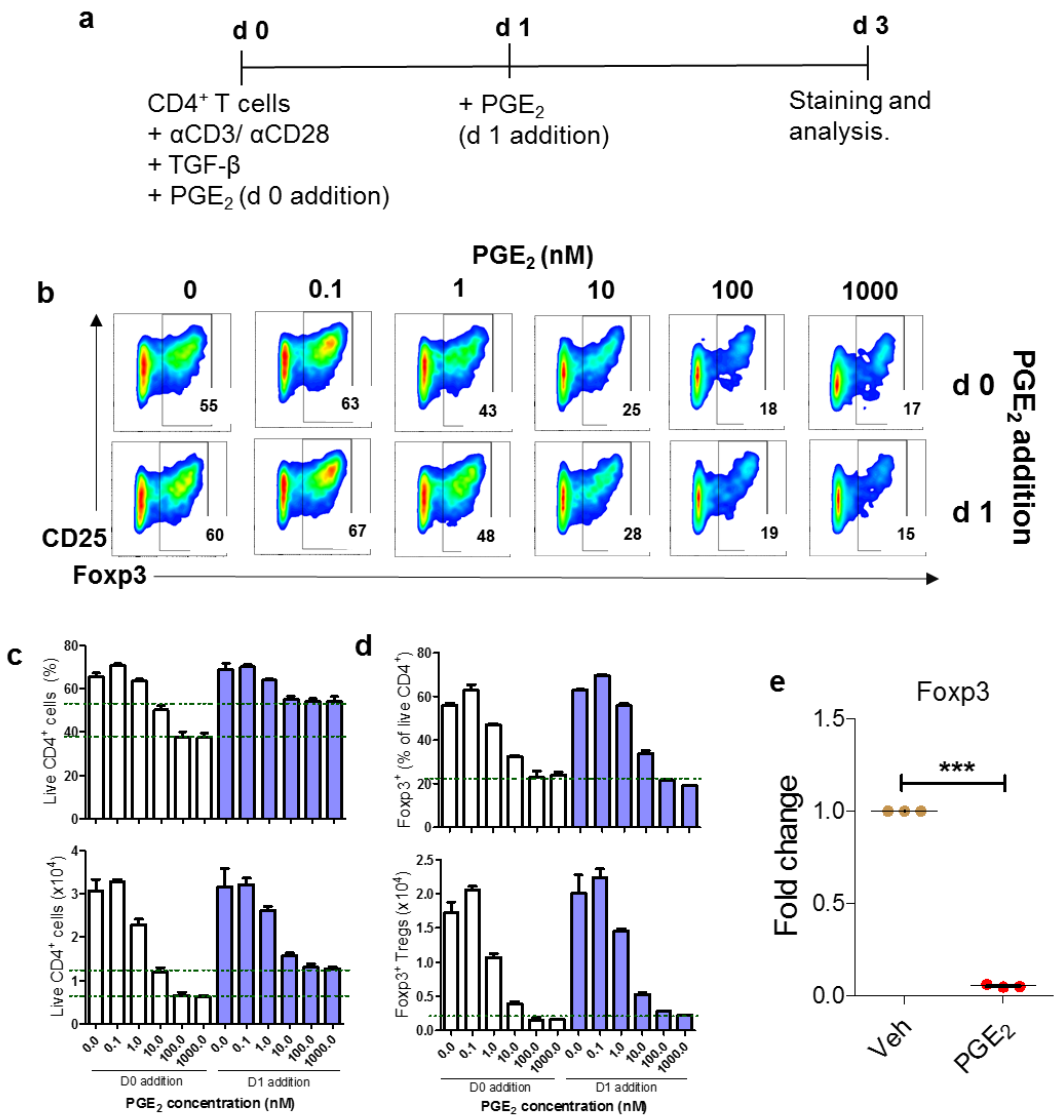


**Figure 3.2: The effect of postponed $PGE_2$ addition on Foxp3 expression**. *(a) Experimental timeline for an in vitro cell culture. Freshly isolated CD4⁺ T cells were simulated with αCD3/αCD28 and TGF-β in the absence or presence of indicated concentrations of $PGE_2$ added either on d 0 or d 1. On d 3, cells were harvested and intracellularly stained with anti-mouse Foxp3. (b) Representative dot plots show Foxp3 expression gated on live CD4⁺ cells. (c) Percentages and numbers of live CD4⁺ T cells under indicated conditions. (d) Percentages and numbers of Foxp3⁺ cells within total live CD4⁺ T cells under indicated conditions. (e) Sorted naïve CD4⁺CD25⁻Foxp3-YFP⁻ T cells cultured with vehicle or PGE2 (100 nM) added on day 1 were harvested day 3 and processed for PCR. Foxp3 expression was detected. (Mean ± SEM of triplicates). Data represents one experiment. Representative of 3 independent experiments.*

### 3.3.3 $PGE_2$ negatively affects natural Treg $CD25^+Foxp3^+$ expression

Previous experiments had used AutoMACs sorted $CD4^+$ cells, that may have included both naïve T cells and nTregs, therefore spleens from Foxp3-YFP mice were sorted for nTregs ($CD4^+CD25^+$Foxp3-$YFP^+$). Cells were cultured with αCD3, αCD28 and TGF-β for 24 hours prior to addition of $PGE_2$ on day 1. Day 3, cells were harvested and stained for Treg markers (**Fig. 3.3a**). Flow cytometry dot plots of CD25 and Foxp3 expression in live nTregs cultured with vehicle or $PGE_2$ (**Fig. 3.3b**). nTregs cultured with $PGE_2$ saw a reduction in cells expressing both CD25 and Foxp3, whereas total Foxp3 was similar (**Fig. 3.3c**). Cells were also processed for PCR. Unlike in whole T cells (**Fig. 3.2f**), *Foxp3* gene expression was largely unaffected in $PGE_2$ cultured nTregs compared to the vehicle group (**Fig. 3.3d**). This suggests that $PGE_2$ is affecting cell activation and induction of Foxp3 expression.

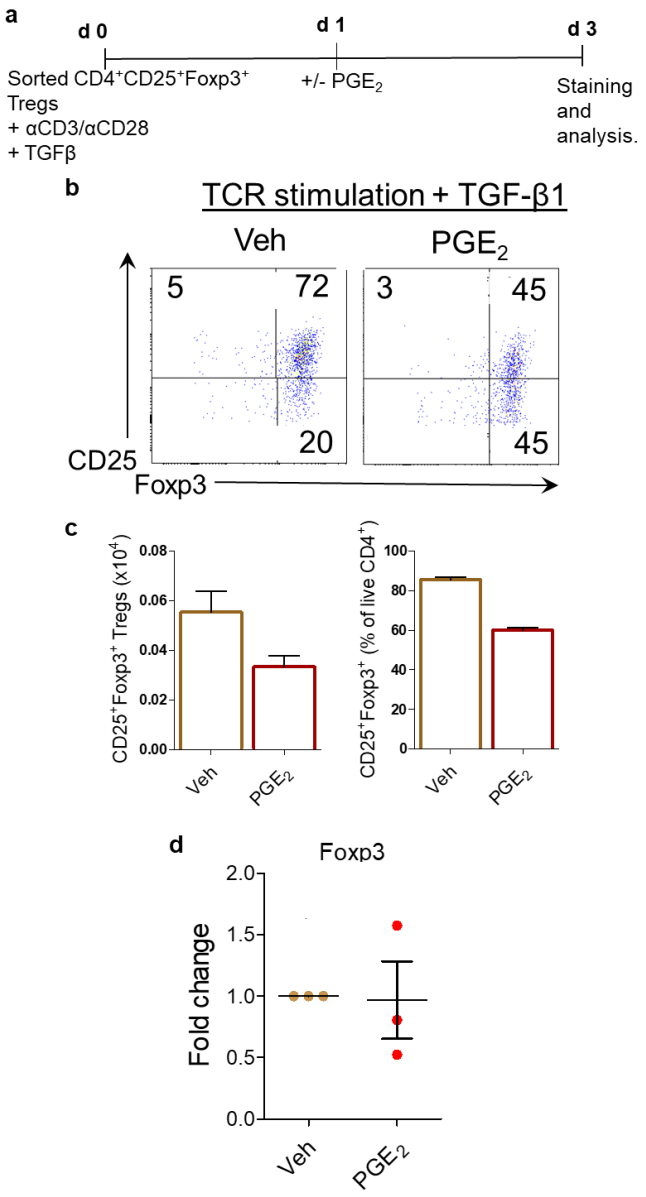


**Figure 3.3: The effect of $PGE_2$ on nTreg Foxp3 expression.** *(a) Experimental timeline for in vitro cell culture. Freshly isolated CD4 isolated $CD4^+CD25^+Foxp3^+$ nTregs were simulated with αCD3/αCD28 and TGF-β in the absence or presence of indicated concentrations of $PGE_2$ on d 1. On d 3, cells were harvested and intracellularly stained with anti-mouse Foxp3. (b) Flow cytometry dot plot of CD25 and Foxp3 expression in live $CD4^+$ T cells. (c) Percentage and number of $CD25^+Foxp3^+$ Tregs cultured with either vehicle or $PGE_2$. (d) Foxp3 gene expression fold change between nTregs cultured with either vehicle or $PGE_2$. (Mean ± SEM of triplicates). Data represents one experiment. Representative of 3 independent experiments.*

### 3.3.4 $PGE_2$ inhibits Foxp3 induction via EP2 and EP4 receptors

$PGE_2$ has four receptor subtype (EP1-4) which can each activate different downstream signalling pathways, and have varied affinities for $PGE_2$ which was discussed in chapter 1. EP2 and EP4 have been shown to be involved in induction of Th1 and Th17, therefore the involvement of these EP receptors in suppressing Foxp3 expression was examined.

To detect this, freshly isolated $CD4^+$ T cells were stimulated with αCD3/αCD28 and TGF-β on day 0, $PGE_2$ analogues, EP agonists and antagonists were added 24 hours later. A $PGE_2$ analogue (17-phenyl trinor $PGE_2$) which preferably binds to EP1/EP3 receptors, an EP2 and EP4 selective agonist and an EP2 or EP4 selective antagonist were used. Cells were harvested and Foxp3 expression was detected by flow cytometry on day 3 (**Fig. 3.4a**).

It was observed that $PGE_2$-induced Foxp3 suppression was overcome by the combined EP2 and EP4 antagonists (**Fig. 3.4b, c, d**). The EP4 antagonist alone could also partially rescue Foxp3 expression, but EP2 antagonist alone had no effect (**Fig. 3.4b, c, d**). These data suggest that EP2 and EP4 receptors are vital in $PGE_2$'s suppression of Foxp3 expression.

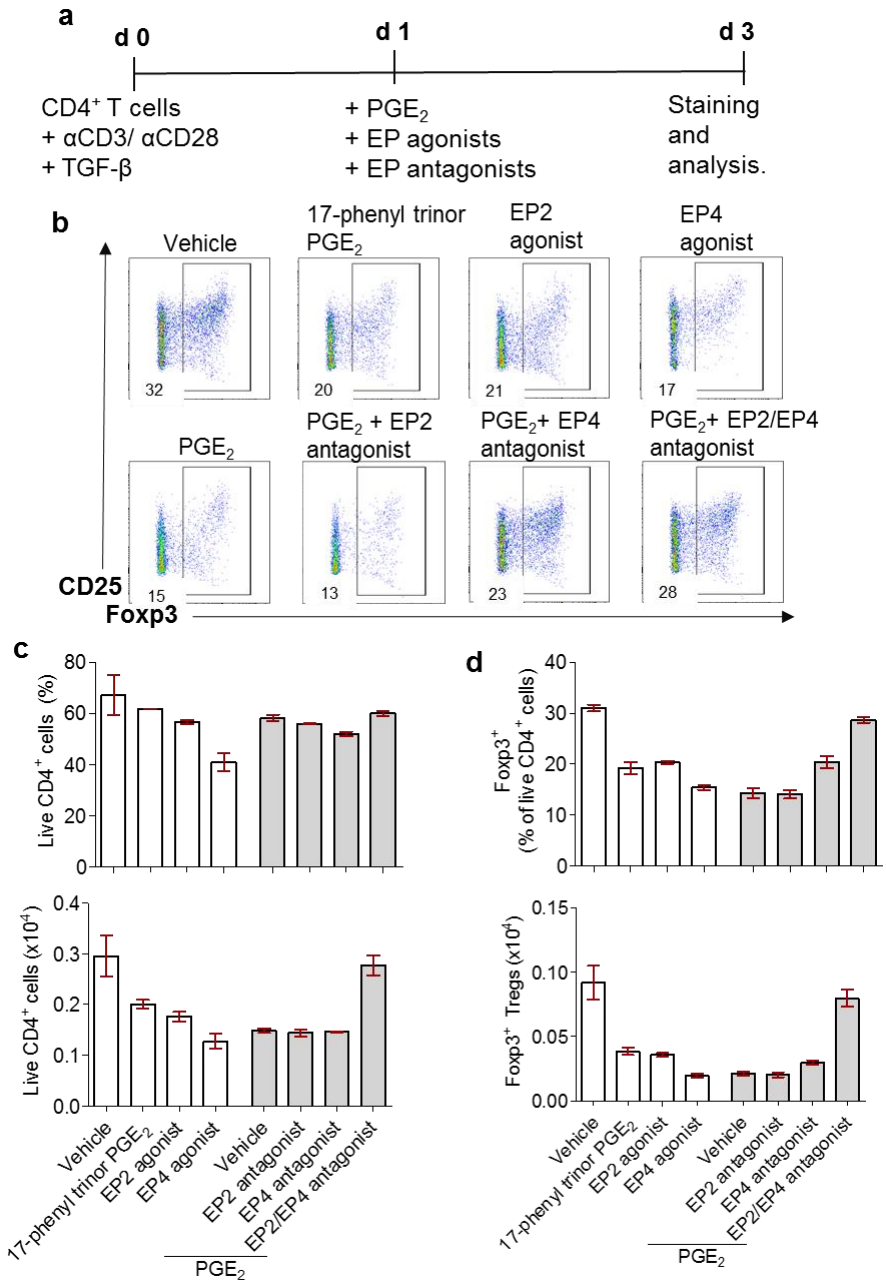


**Figure 3.4: The role of EP2 and EP4 in suppression of Foxp3 expression *in vitro*. (***a*) *Experimental timeline for in vitro cell culture. Freshly isolated CD4⁺ T cells were stimulated with αCD3/αCD28 and TGF-β on d 0. $PGE_2$, EP agonists and EP antagonists were added 24 hours after T cell activation. On d 3, cells were harvested and intracellularly stained with anti-mouse Foxp3. (b) Flow cytometry dot plots of Foxp3 expression in live CD4⁺ T cells on d 3. (c) Number and percentage of live CD4⁺ cells under indicated conditions. (d) Number and percentages of Foxp3⁺ cells within total live CD4⁺ T cells under indicated conditions (mean ± SEM of triplicates). Data represents one experiment. Representative of **3** independent experiments.*

### 3.3.5 The role of the cAMP pathway in suppression of Foxp3 expression

It was observed that inhibition of EP2 and EP4 prevented $PGE_2$'s suppression of Foxp3 expression, and this was largely dependent on the EP4 antagonist, illustrating the two key receptors for transmitting the $PGE_2$ signal in the context of Foxp3 inhibition. Therefore, mechanisms for $PGE_2$'s suppression of Foxp3 was examined through manipulation of various signal pathways. The main downstream signal activated by EP2 and EP4 receptors is the cAMP-PKA pathway, so this was the first route manipulated to see whether it was involved in Foxp3 expression.

Freshly isolated $CD4^+$ T cells were stimulated with αCD3/αCD28 and TGF-β on day 0, then $PGE_2$ and the following compounds; a cAMP analog, dibutyryl-cAMP (db-cAMP), and a non-selective phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) were added 24 hours later. On day 3, cells were harvested and Foxp3 expression was detected using flow cytometry (**Fig. 3.5a**).

Similar to $PGE_2$, db-cAMP dose dependently suppressed TGF-β-induced Foxp3 and expression (**Fig**. **3.5b, c**). IBMX, which enhances intracellular cAMP by preventing degradation, also mimicked Foxp3 suppression (**Fig. 3.5b, c**). Protein kinase A (PKA) is downstream of cAMP, therefore an inhibitor H-89 was used to prevent further transition of this signal. $PGE_2$ suppressed TGF-β-induced Foxp3 expression as observed previously, however notably this suppression could be partially rescued by blockade of PKA activity (**Fig. 3.5d, e**). These data indicated that $PGE_2$ suppresses TGF-β-induced Foxp3 expression through the cAMP-PKA pathway.

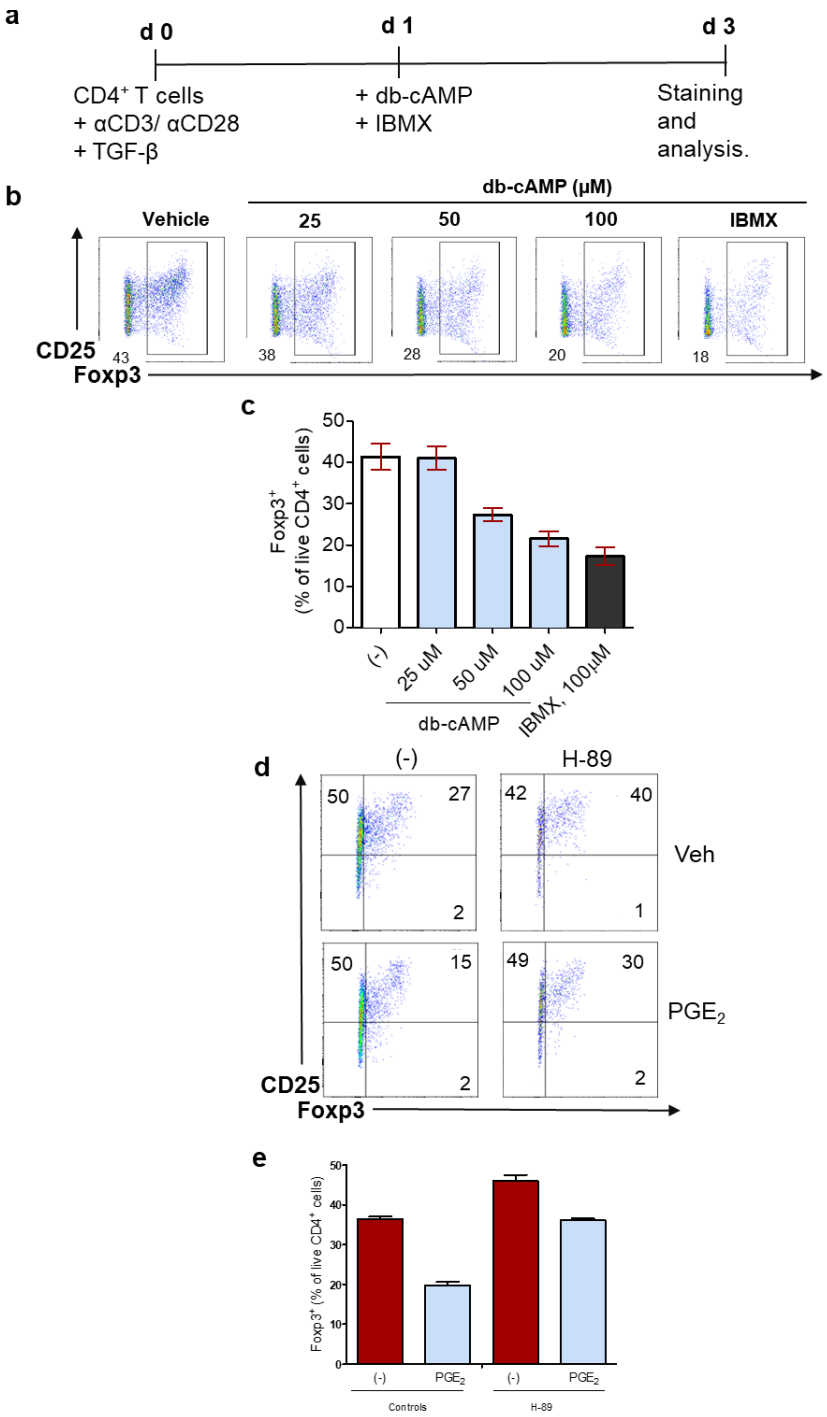


**Figure 3.5: The effect of cAMP-PKA pathway on Foxp3 expression**. *(a) Experimental timeline for in vitro cell culture. Freshly isolated CD4⁺ T cells were stimulated with αCD3/αCD28 and TGF-β on d 0. Various concentrations of db-cAMP and IBMX (100 uM) were added 24 hours after T cell activation. On d 3, cells were harvested and intracellularly stained with anti-mouse Foxp3. (b) Expression of Foxp3 in live CD4⁺ T cells on d 3. Representative dot plots were gated on live CD4⁺ cells. (c) Bar graphs show percentages of live Foxp3⁺ cells within total live CD4⁺ T cells under indicated conditions. (d) Expression of CD25 and Foxp3 on*

*d 3 in live CD4$^+$ T cells stimulated with TGF-β and in the absence or presence of PGE2 or H-89. Representative dot plots were gated on live CD4$^+$ cells. (e) Bar graphs show percentages of live Foxp3$^+$ cells within total live CD4$^+$ T cells under indicated conditions (mean ± SEM of triplicates). Representative of three independent experiments.*

### 3.3.6 LY294002, a PI3K inhibitor, could partially rescue $PGE_2$-induced Foxp3 suppression

Besides the cAMP-PKA pathway, PI3K can similarly be activated by $PGE_2$ signaling through EP2 and EP4 in T cells [87, 161]. It was then questioned whether PI3K is involved in PGE2-mediated suppression of Foxp3 through use of Ly294002, a PI3K inhibitor. To address this, T cells were stimulated with αCD3/αCD28 and TGF-β on day 0, and added $PGE_2$ with or without LY294002, 24 hours later. Foxp3 expression was detected by flow cytometry on day 3 (**Fig. 3.6a**).

While LY294002 itself did not affect TGFβ-induced Foxp3 expression or cell viability, it could partially rescue $PGE_2$-dependent suppression of Foxp3 expression (**Fig. 3.6b, c**). These data suggest that PI3K may also be involved in $PGE_2$ suppression of Foxp3 induction, but more evidence should be obtained using other approaches, for example other downstream inhibitors.

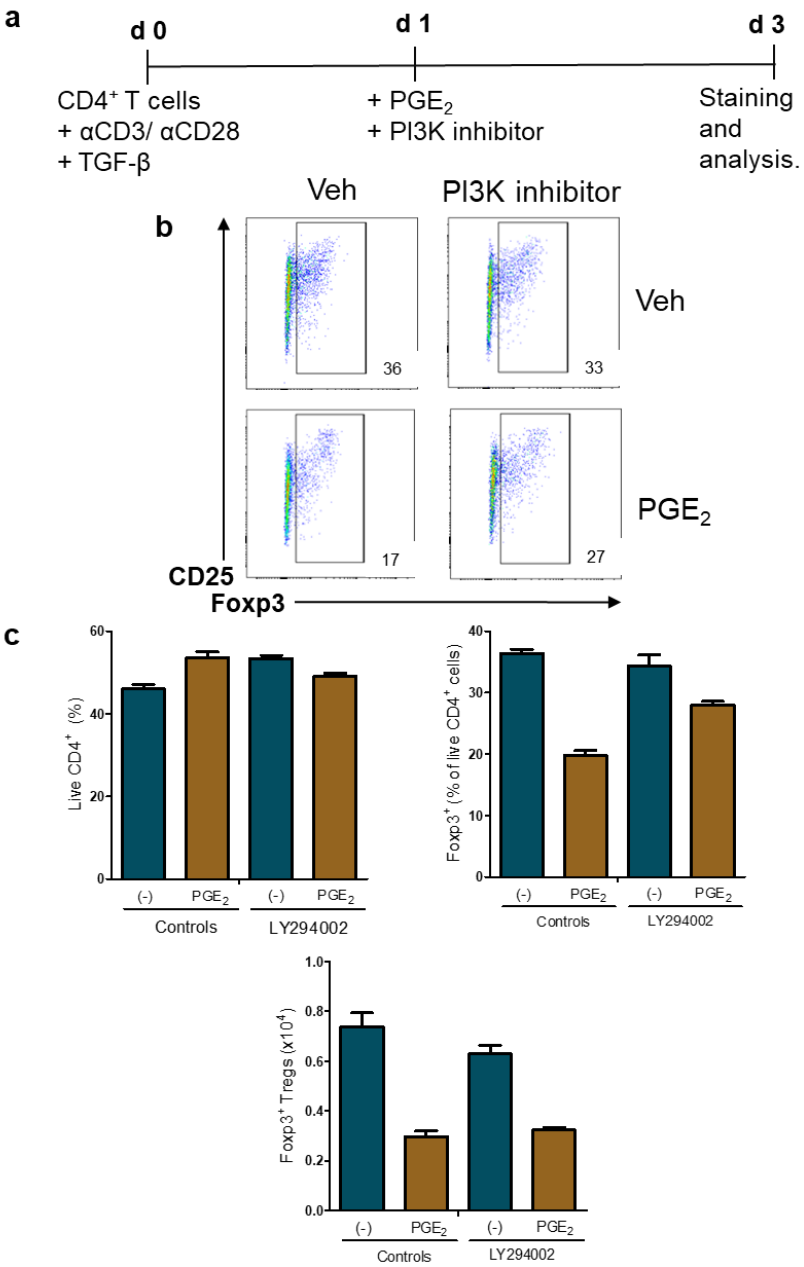


**Figure 3.6: The effect of PI3K on Foxp3 expression.** *(a) Experimental timeline for an in vitro cell culture. Freshly isolated CD4⁺ T cells were stimulated with αCD3/αCD28 and TGF-β on d 0. $PGE_2$ and/or LY-294002 were added 24 hours after T cell activation. On d 3, cells were harvested and intracellularly stained with anti-mouse Foxp3. (b) Foxp3 expression in live CD4⁺ T cells on d 3. Representative dot plots were gated on live CD4⁺ cells. (c) Bar graphs show percentages of live CD4⁺ and Foxp3⁺ cells within total live CD4⁺ T cells under indicated conditions (mean ± SEM of triplicates). Representative of three independent experiments.*

## 3.4 Discussion

In summary, these initial *in vitro* experiments demonstrated that $PGE_2$ down-regulates the TGF-β response in $CD4^+$ T cells thus preventing $Foxp3^+$ expression and iTreg differentiation. This inhibitory effect of $PGE_2$ on Foxp3 expression is mediated by the receptor subtypes EP2 and EP4 and their down-stream cAMP/PKA and PI3K pathway. $PGE_2$ suppression of TGF-β-induced Foxp3 expression in T cells could be due to its disruption of TCR signalling and reduction of IL-2 production, evidenced as reduction of total cell viability [87]. This possibility was then excluded as postponed addition of $PGE_2$ partially rescued $PGE_2$'s negative influence on T cell viability and activation, but did not influence $PGE_2$ suppression of Foxp3 expression.

**Figures 3.1 and 3.2** demonstrate that $PGE_2$ addition markedly suppressed TGF-β-induced Foxp3 expression in the murine $CD4^+$ T cells. $PGE_2$ acts via disruption of TCR signalling and reducing IL-2 expression, therefore it is not unexpected that addition of PGE2 reduced cell viability (**Fig. 3.1**) [87]. Postponed addition of $PGE_2$ could partially rescue $PGE_2$'s negative influence on T cell viability and activation, but had no effect on $PGE_2$ suppression of Foxp3 expression (**Fig. 3.2**). This excludes the possibility that $PGE_2$ suppression of Foxp3 expression is a secondary effect from its suppression of T cell activation or survival.

This is in contrast with other literature which suggests Foxp3 levels increase in the presence of $PGE_2$, for example Dubinett's group found increased Foxp3 expression when human T cells were stimulated with $PGE_2$ [111]. Dubinett's group used $PGE_2$ at both 13 and 26 μM which is higher than would be present physiologically, whereas we had shown the effect of $PGE_2$ negatively influencing Treg development at 10, 100 and 1000 nM which is more physiologically relevant [111]. This is especially important in *in vitro* cell cultures, where $PGE_2$ is unlikely to be broken down quickly enough as it would be *in vivo* [88]. Additionally, Baratelli. F, *et al* (2012) stated that $PGE_2$ induced Foxp3 expression after culturing human naïve T cells with supernatant from COX-2 overexpressing lung cancer cells [111]. Although this supernatant will contain COX-2, there will also be a multitude of other factors present that can influence Foxp3 expression.

However, these findings were contrasted by work from Chen, H. *et al* (2009), who similarly observed dose-dependent suppression of mRNA *Foxp3* expression in $CD4^+CD62L^+$ naïve murine T cells after treatment with $PGE_2$ compared to TGFβ only [159]. Dr Yao has also done preliminary work on human cells, and a genomic microarray of these cells correlated with the murine work (data unpublished).

It was observed that naïve T cells cultured with $PGE_2$ had reduced Foxp3 gene expression, whereas the activation status (CD25 expression) of nTregs was affected but Foxp3 expression was unaffected (**Fig. 3.3**). The reason why there is a reduction in Foxp3 expression observed with flow cytometry however this is not seen with gene expression possibly because flow cytometry data is gated on 'current' live $CD4^+$ T cells, whereas nTregs processed for PCR were initially sorted for live nTregs, however not re-sorted for live cells prior to observing gene expression. This is due to the low number of cells that would be recovered, thus potentially these cells are a mixture of live and dead Foxp3 expressing nTregs, additionally it does not show change of CD25 (activation status) that the flow cytometry data also provides. Baratelli *et al* (2012) similarly observed a reduction in Treg CD25 expression when culturing cells with $PGE_2$ [111]. This suggests that $PGE_2$ affects Treg activation.

Suppression of Foxp3 was mimicked by EP2 and EP4 agonists and this suppression was prevented by combination of EP2 and EP4 antagonists (**Fig. 3.4**). This suggests that both EP2 and EP4 are important for Foxp3 expression, relevant because these receptors have also been shown to enhance the proliferation and differentiation of Th1 and Th17 cells, the antithesis of Tregs [102, 103]. This is supported by work by Esaki, Y *et al* (2010), who demonstrated that use of an EP4 antagonist greatly reduced EAE symptoms in mice compared to the control group, and disease severity was further suppressed in $EP2^{-/-}$ mice treated with an EP4 antagonist, demonstrating the importance of these two key $PGE_2$ receptors in induction of pro-inflammatory Th1 and Th17 cells [137]. This may due to an increased number of $Foxp3^+$ cells, suppressing the inflammatory environment.

The cAMP-PKA pathway has been implicated in induction of Foxp3 expression, increased levels of CREB activating the transcription factor ATF [30]. Upon Treg activation, cAMP levels are upregulated to prevent proliferation and IL-2 expression in inflammatory cells [162, 163]. However, it is known that $PGE_2$ stimulation elevates intracellular cAMP via EP2 and EP4, therefore it was examined what effect addition of increased concentrations of a cAMP analog were added to naïve T cells to see whether this influenced Foxp3 expression [30, 91]. Interestingly, the cAMP analog dose-dependently suppressed TGF-β induced Foxp3 expression similar to what was observed with a dose-response of $PGE_2$ in the *in vitro* cell culture. This was mirrored by Foxp3 expression levels observed after naïve T cells were incubated with IBMX, a competitive nonselective phosphodiesterase inhibitor which raises intracellular cAMP (**Fig. 3.5**). This is supported by recent data which suggests that activation of the cAMP pathway, inducing the transcription factor CREB, negatively effects $Foxp3^+$ Tregs [33, 34] .

By contrast, inhibition of PKA, downstream of cAMP, by H-89 prevented $PGE_2$ suppression of Foxp3 (**Fig. 3.5**). Klein, M. *et al* (2012, 2016) demonstrated that cAMP inhibition should reduce Tregs suppressive ability, therefore testing the suppressive function of these cells under different conditions, such as increased db-cAMP, H-89, or with $PGE_2$ merits further investigation [162, 163]. This work suggests that $PGE_2$ suppresses Foxp3 via the cAMP-PKA pathway, as with increased levels of the cAMP analogues, there is reduced Foxp3 expression, and use of H-89 was able to partially rescue $PGE_2$-mediated Foxp3 suppression.

The PI3K/AKT pathway can also be activated by EP2 and EP4 stimulation. Yao, C. *et al* (2009) demonstrated that blockade of PI3K signalling could prevent $PGE_2$'s stimulated IFNɣ production in Th1 cells [163]. PI3K signalling is known to inhibit Foxp3 expression via activation of AKT which inhibits the transcription factor Foxo [30, 31, 164, 165]. Novel inhibitors of which are being developed for anti-tumour immunity [165]. These results indicated that the PI3K inhibitor LY294002 did not affect TGF-β-induced Foxp3 expression, but it did partially rescue $PGE_2$-dependent suppression of Foxp3 (**Fig. 3.6**). These results suggest that PI3K mediates $PGE_2$'s action in both Th1 and Treg cells.

## 3.5 Conclusion

This work demonstrated that $PGE_2$ reduced Foxp3 expression in TGF-β-induced *in vitro* Treg differentiation, and this involves the cAMP-PKA pathway. All this work was carried out in an *in vitro* situation; therefore it was vital to observe the effect of $PGE_2$ inhibition *in vivo* to confirm these findings.

# 4 Endogenous $PGE_2$ alters the Tissue Resident Treg Population

## 4.1 Introduction

In the following chapter, to see whether inhibition of $PGE_2$ *in vivo* influenced immune cells and whether or not this was the same effect in different organs, cell populations within the colons, spleens and mesenteric lymph nodes (mLNs) were examined. These tissues were chosen because the spleen and mLNs are both important lymphoid organs for lymphocyte production and activation respectively and are therefore useful to determine the role of $PGE_2$ in immune effector cell development. The colon was selected because it contains a greater number of immune cells than other tissues due to the continuous exposure to a plethora of dietary antigens and gut microbiota, which are involved in stimulating immune responses. Additionally, as $PGE_2$ can be either protective or damaging depending on the cells affected, it was of interest to see what effect it had on Tregs within the gut [166].

The intestines are a potentially inflammatory environment due to the microbes and food antigens amongst other factors that interact with the mucosa layer. Hence the co-evolution of the intestinal immune system alongside the commensal microbiota is important to prevent excessive inflammation. Within this environment, tTregs, or naïve T cells that migrate to the colonic environment prior to differentiation into pTregs, suppress pro-inflammatory cells via multiple mechanisms, such as antagonising pro-inflammatory effector T cells, through contact-dependent mechanisms such as Cytotoxic T-Lymphocyte-Associated protein-4 (CTLA-4), and release of soluble cytokines such as IL-10 and TGF-β [3]. Sakaguchi. S, *et al* (1995) demonstrated that removal of these cells elicited an autoimmune response to certain self-antigens which confirmed their importance in maintaining tolerance [3, 13].

Within the colonic pTreg population, approximately 40 – 60 % co-express RORγt, and the extent of co-expression correlates with microbial diversity [55]. These cells are more stable within an inflammatory environment due to significant demethylation at Treg signature genes, such as *Foxp3* and *CTLA-4,* and also have a better flexibility to respond to signals due to the homing molecules CD62L and mTGFβ that are expressed to a greater extent compared to $Foxp3^{+}RORγt^{-}$ Tregs [55, 167].

Previous work has shown how $PGE_2$, via the receptors EP2 and EP4, increases the differentiation of Th1 cells and enhances IL-23 production by APCs to enable Th17 expansion [91, 102]. However, the role that $PGE_2$ plays on the induction of tissue resident regulatory T cells is less clear

In **chapter 3**, it was demonstrated that *in vitro* addition of $PGE_2$ to a Treg culture system resulted in reduced *Foxp3* gene and protein expression, and this was largely dependent on the EP4 receptor. Therefore, to understand the effects of $PGE_2$ on regulation of Tregs *in vivo*, endogenous $PGE_2$ production was inhibited by a non-selective COX inhibitor indomethacin (a NSAID) and $Foxp3^+$ Treg cells were analysed in various organs. COX inhibitors inhibit the activity of the COX enzymes by binding to the active site, and block arachidonic acid from forming a complex therefore prevents further downstream prostaglandin products from being formed [4]. To further study the role of $PGE_2$-EP4 signalling, an EP4 selective agonist (L-902,688) was also used [112].

## 4.2 Experimental methodology

To determine the effect of inhibiting $PGE_2$ *in vivo* on murine tissue Tregs, WT C57BL/6 mice were treated with either vehicle (0.5 % ethanol) or a non-steroidal inflammatory drug (NSAIDS) indomethacin (5 mg/kg/day) to inhibit $PGE_2$ production, or indomethacin plus an EP4 agonist (L-902,688) to specifically activate this key $PGE_2$ receptor. In addition to this, as indomethacin is known to potentially cause intestinal inflammation, a low dose (refer to **section 2.8.1** in the methods chapter for doses) long-term treatment of indomethacin was employed to confirm that the results observed were due to $PGE_2$ inhibition, not the treatment dose concentration.

Spleen, mLNs and colons were removed from mice following treatment with vehicle or indomethacin, and processed into a single cell suspension (detailed protocols available in **section 2, 2.31, 2.3.2, 2.3.3**). RBCs were lysed then cells stained for tissue resident Tregs using the following panels for flow cytometry. Single cells were stained for live/dead (ef780), CD45 (APC-Cy7, efluor450), CD4 (PerCP-Cy5.5, PE), CD3 (AF700, PE, APC), CD25 (APC, BV605, FITC), Foxp3 (FITC, PE) and RORγt (PerCp-eF710).

To boost cytokine expression prior to staining for effector cell types; phorbol 12-myristate 13-acetate (PMA), ionomycin and a Golgi Plug inhibitor were added to the single cell suspension re-suspended in culture medium. Cells were incubated for four hours at 37ºC, 5 % $CO_2$. Cells were resuspended and washed twice with PBS/2 % FCS before being stained with the live/dead marker for 30 mins and incubated at 4ºC. Cells were fixed and permeabilised overnight before staining for cytokines and other cellular markers for one hour. Cells were washed and prepared for flow cytometry. The panel to determine the effector cell type was; live/dead (ef780), CD45 (APC-Cy7, efluor450), CD4 (PerCP-Cy5.5, PE), CD3 (AF700, PE, APC), CD25 (APC, BV605, FITC), Foxp3 (FITC, PE) to determine the negative population, IFNɣ (FITC), IL-17a (PerCP-Cy5.5) and Ly6G (APC). Samples were all run on the BD LSR 5 laser Fortessa (BD bioscience).

Treg levels were also examined in tissues from WT or EP4 T cell specific knockout mice under steady-state conditions, to see the effect of inhibition of EP4 signalling on endogenous Treg levels. Tissues were processed as previously described into a single cell suspension and stained using the previously described Treg staining panel for flow cytometry analysis.

Mouse tissue extraction was performed in accordance to U.K. Home Office legislation. Mice were analysed individually and none were excluded from the analysis, with the exception of exclusions due to technical errors in preparation of intestinal lamina propria lymphocytes (LPLs).

### 4.2.1 Aims of chapter

- To determine the role of $PGE_2$ on tissue resident Tregs.
- To determine whether $PGE_2$ inhibition of Foxp3 is T cell dependent.

## 4.3 Results

### 4.3.1 $PGE_2$-EP4 signalling suppresses $Foxp3^+$ Tregs *in vivo*.

To study the effect of $PGE_2$ on Tregs *in vivo*, WT C57BL/6 mice were treated for five days with vehicle (0.5 % ethanol), indomethacin (5 mg/kg/day), a NSAID to block endogenous $PGE_2$ production, or indomethacin plus L-902,688 (an EP4 agonist), to specifically activate the $PGE_2$ receptor EP4. On day 5, Tregs in various tissues were analysed by flow cytometry (**Fig. 4.1a**). The gating strategy for analysis of $Foxp3^+$ Tregs among live $CD4^+$ cells is shown in **Fig. 4.1b**.

Supporting previous *in vitro* work presented in chapter 3, inhibition of endogenous PG production resulted in a greater number and proportion of $Foxp3^+$ Tregs compared to the vehicle treated group within the colonic lamina propria ($p \leq 0.0001$), spleen ($p \leq 0.001$) and mLNs ($p \leq 0.001$) (**Fig. 4.1c - f**). The increase in Foxp3 expression induced by indomethacin was prevented by co-treatment with an EP4 agonist, suggesting that activation of the $PGE_2$-EP4 signalling pathway suppressed $Foxp3^+$ Treg induction at the steady state (**Fig. 4.1c - f**). The indomethacin treated mice had a significantly greater number of colonic $Foxp3^+$ Tregs compared to mice treated with vehicle, or indomethacin plus an EP4 agonist ($p \leq 0.05$). This was not as clear within the mLNs or spleen, suggesting that $PGE_2$ - EP4 signalling selectively suppresses Tregs in the colonic environment (**Fig. 4.1f**). The number of T conventional cells ($CD4^+Foxp3^-$ cells) were unaffected by treatment in the various organs (**Fig. 4.1g**). This data suggests that $PGE_2$-EP4 signalling suppresses intestinal Tregs *in vivo*.

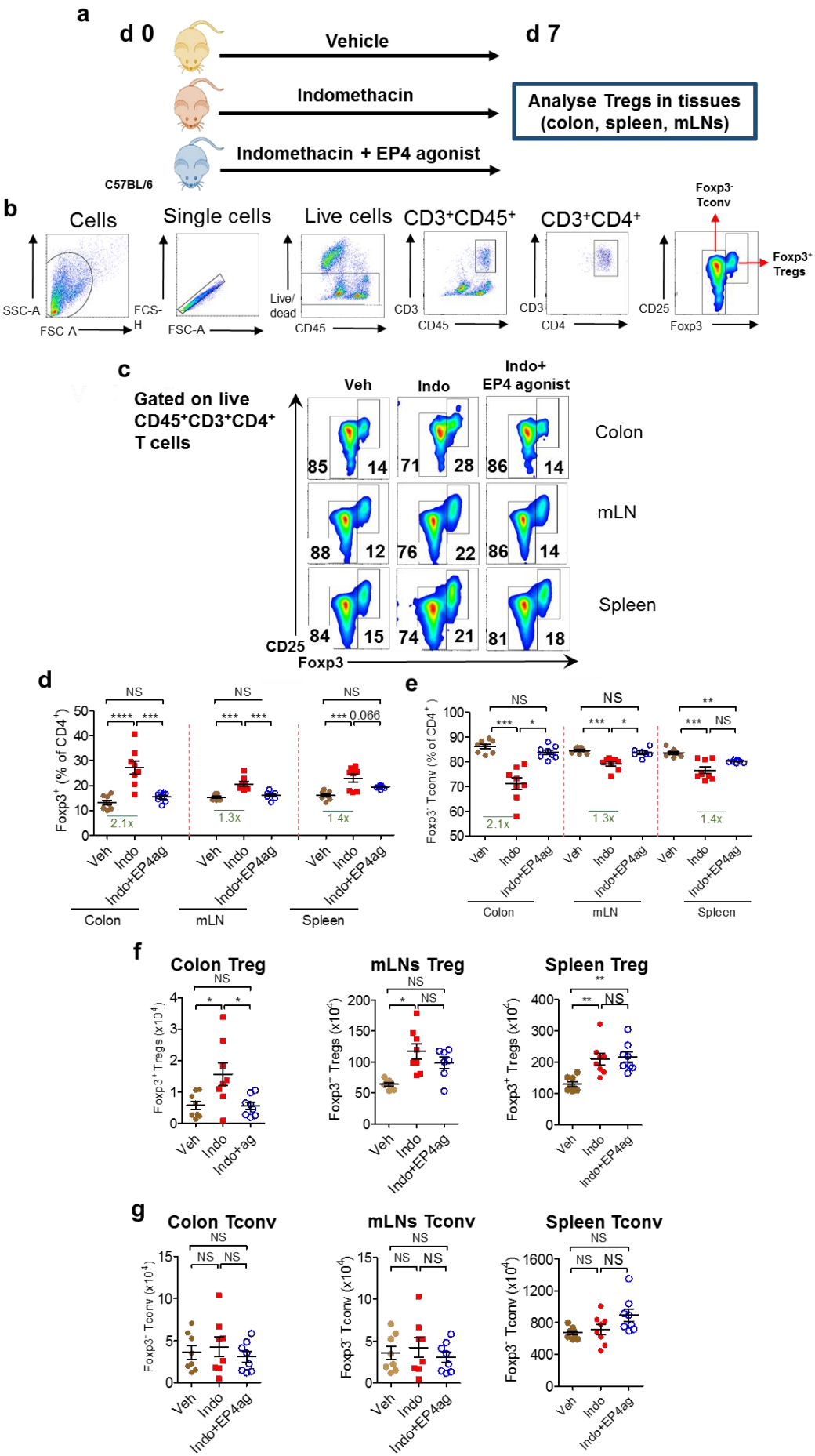
a
d 0
Vehicle
d 7
Indomethacin
Analyse Tregs in tissues (colon, spleen, mLNs)
Indomethacin + EP4 agonist
C57BL/6
b
Cells
Single cells
Live cells
CD3+CD45+
CD3+CD4+
Foxp3- Tconv
Foxp3+ Tregs
SSC-A
FSC-A
FCS-H
Live/ dead
CD45
CD3
CD4
CD25
Foxp3
c
Gated on live CD45+CD3+CD4+ T cells
Veh
Indo
Indo+ EP4 agonist
Colon
mLN
Spleen
85 14
71 28
86 14
88 12
76 22
86 14
84 15
74 21
81 18
d
Foxp3+ (% of CD4+)
2.1x
1.3x
1.4x
e
Foxp3- Tconv (% of CD4+)
f
Colon Treg
mLNs Treg
Spleen Treg
Foxp3+ Tregs (x10^4)
g
Colon Tconv
mLNs Tconv
Spleen Tconv
Foxp3- Tconv (x10^4)
Veh
Indo
Indo+EP4ag
NS

**Figure 4.1: Effect of $PGE_2$ on tissue resident $Foxp3^+$ Tregs *in vivo.*** *(a) Experimental timeline for in vivo inhibition of endogenous $PGE_2$. WT C57BL/6 mice were treated with either vehicle, or indomethacin in drinking water for 7 days, or indomethacin plus an EP4 agonist, delivered by i.p injection daily. Mice were culled on d 7, tissues removed and processed to a single cell suspension by* macerating the spleen, mLNs and digesting colonic tissue*. Cells were stained with anti-CD45, anti-CD3, anti-CD4, anti-CD25, anti-Foxp3 antibody. (b) Flow cytometry plot illustrates the gating strategy for Foxp3 and CD25 expression in live $CD4^+$ T cells from colonic tissue. (c) Expression of CD25 and Foxp3 in live $CD4^+$ T cells in the spleen, mLNs and colons of mice treated with either vehicle, indomethacin, or indomethacin plus an EP4 agonist. Representative dot plots were gated on live $CD4^+$ cells. (d) Percentages of $Foxp3^+$ Tregs within total live $CD4^+$ cells in spleens, mLNs and colons of mice treated under different conditions. (e) Percentages of $Foxp3^-$ Tconvs within total live $CD4^+$ cells in spleens, mLNs and colons of mice treated under different conditions. (f) Total number of $Foxp3^+$ cells within total live $CD4^+$ cells in spleens, mLNs and colons of mice treated under different conditions. (g) Total number of $Foxp3^-$ cells within total live $CD4^+$ cells in spleens, mLNs and colons of mice treated under three different conditions. Data shown as means ± SEM (error bars) are pooled from two independent experiments. (n = 8) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ by Kruskal-Wallis test, Dunn's Multiple Comparison test used for post-hoc comparison.*

### 4.3.2 $PGE_2$-EP4 signalling inhibits colonic RORγt$^+$Foxp3$^+$ Tregs at a steady state

Within the intestine, there are a subpopulation of Foxp3$^+$ pTregs (40 – 60 %) that also express the Th17 transcription factor RORγt which is not largely expressed within the tTregs population [55]. These RORγt expressing Tregs are thought to be essential within a pro-inflammatory environment such as the colon, which is constantly in contact with potentially inflammatory factors such as the commensal microbiota [55]. This subset of cells is highly stable and unlikely to have their phenotype switched to a more pro-inflammatory type in the presence of highly inflammatory cytokines, their suppressive ability is greater due to the homing molecules CD62L and mTGFβ they express [55, 167].

Inhibition of endogenous $PGE_2$ *in vivo* resulted in significantly enhanced colonic Foxp3$^+$ Tregs, I thus wondered whether $PGE_2$ also affected the RORγt$^+$ Treg subset. Flow cytometry dot plot of RORγt expression within a Foxp3$^+$ Treg or Foxp3$^-$ Tconv cell population is shown in **Figure 4.2a**. Indeed indomethacin increased RORγt$^+$Foxp3$^+$ Tregs by within the colonic tissue ($p \leq 0.0001$), and this increase was prevented by activation of the EP4 receptor ($p \leq 0.0001$) (**Fig. 4.2b**). In contrast, a smaller proportion of Tregs expressed RORγt in the mLNs and spleens, and these were not influenced by indomethacin or an EP4 agonist treatment (**Fig. 4.2b**). However, RORγt$^+$Foxp3$^-$ T conventional cells, which are expected to be the pro-inflammatory Th17 cells, within these organs including the colon were not affected by indomethacin, or the EP4 agonist (**Fig. 4.2c**). There was similarly an increase in RORɣt$^+$ Treg numbers in colons ($p \leq 0.001$) and mLNs ($p \leq 0.01$) of mice treated with indomethacin, compared to vehicle-treated mice, prevented by EP4 agonist addition, however this was not observed within the spleen (**Fig. 4.2d**). There was no increase in RORɣt$^+$ Tconv cell numbers within the tissue with either indomethacin or indomethacin plus EP4 agonist treated mice (**Fig. 4.2e**). These data suggests that the $PGE_2$-EP4 signalling pathway critically controls the development of the RORγt$^+$ Treg subset in the colon at the steady state.

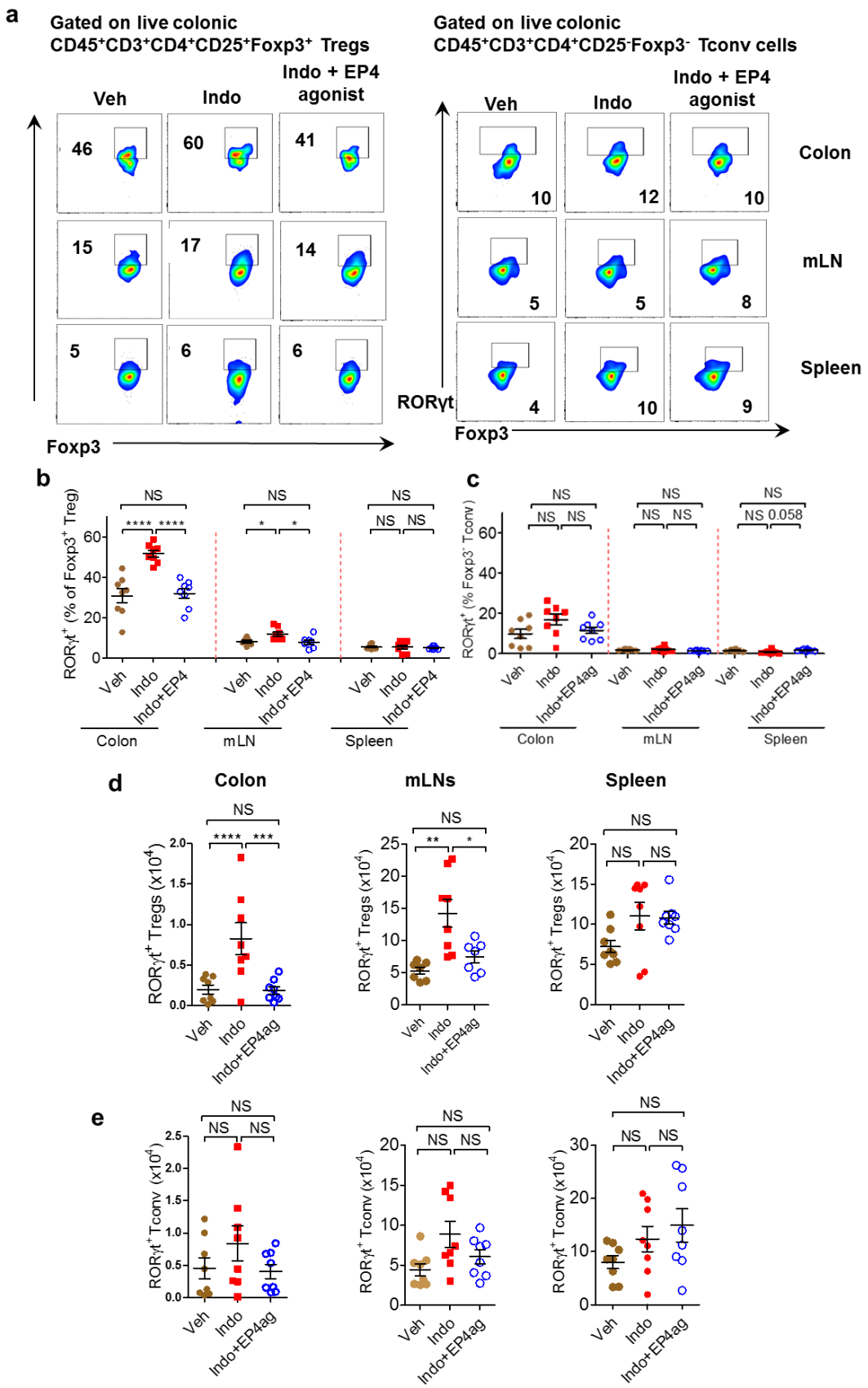
a
Gated on live colonic CD45+CD3+CD4+CD25+Foxp3+ Tregs
Gated on live colonic CD45+CD3+CD4+CD25-Foxp3- Tconv cells
Veh
Indo
Indo + EP4 agonist
Colon
mLN
Spleen
Foxp3
RORγt
b
RORγt+ (% of Foxp3+ Treg)
c
RORγt+ (% Foxp3- Tconv)
Veh
Indo
Indo+EP4
Indo+EP4ag
d
Colon
mLNs
Spleen
RORγt+ Tregs (x10^4)
e
RORγt+ Tconv (x10^4)
NS

**Figure 4.2: Effect of $PGE_2$ on intestinal RORɣt$^+$ Tregs *in vivo*.** *(a) RORɣt expression was measured in Foxp3$^+$ Tregs from **section 4.1.** Flow cytometry dot plot of RORγt and Foxp3 in CD25$^+$Foxp3$^+$ Tregs or Foxp3$^-$ Tconv cells in the spleen, mLNs and colons of mice treated with vehicle, indomethacin or indomethacin plus an EP4 agonist. (b) Percentages of RORγt within CD25$^+$Foxp3$^+$T cells with spleens, mLNs and colons of mice treated under three different conditions. (c) Graphs show percentages of RORγt within Foxp3$^-$ Tconv cells with spleens, mLNs and colons of mice treated under three different conditions. (d) Total number of RORɣt$^+$Foxp3$^+$ Tregs in spleens, mLNs and colons of mice treated under different conditions. (e) Total number of RORγt$^+$ Tconv cells in the spleens, mLNs and colons of mice treated under three different conditions. Data shown as means ± SEM (error bars) are pooled from two independent experiments. (n = 8) * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001 by Kruskal-Wallis test, Dunn's Multiple Comparison test used for post-hoc comparison.*

### 4.3.3 $PGE_2$-EP4 signalling also inhibits small intestine Foxp3$^+$ Tregs at a steady state

It had been shown that only within the colon, an increase in Foxp3$^+$ Tregs, especially the RORɣt$^+$Foxp3$^+$ subpopulation, was observed after inhibition of $PGE_2$ production. It was then wondered whether Foxp3$^+$ Tregs were similarly regulated within the small intestine. This is because differences occur between them due to the changing environment along their length, although the small intestine and colon are part of the digestive tract and have similar morphological features, such as both are involved with the digestion of food. For example, the small intestine is more acidic compared to the colon and therefore has a reduced commensal microbiota diversity compared to the more diverse colon microbial community [51, 55].

As described in **section 4.3.1**, WT C57BL/6 mice were treated with either vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) five days in this experiment. Mice were culled on day 5 and Tregs were analysed within the small intestine by flow cytometry (**Fig. 4.3a**). The flow cytometry dot plot of Foxp3 and RORγt expression in live CD4$^+$ T cells is demonstrated in **figure 4.3b**. There was increased Foxp3$^+$ and RORγt$^+$Foxp3$^+$ expression in the small intestine of mice treated with indomethacin, compared to those treated with vehicle (**Fig. 4.3c, d**). There was also a slight increase in RORγt$^+$Foxp3$^-$ T conventional cells within the small intestine from the indomethacin-treated mouse (**Fig. 4.3e**). These results indicated a similar mechanism for regulation of mucosal Tregs in both colon and small intestine.

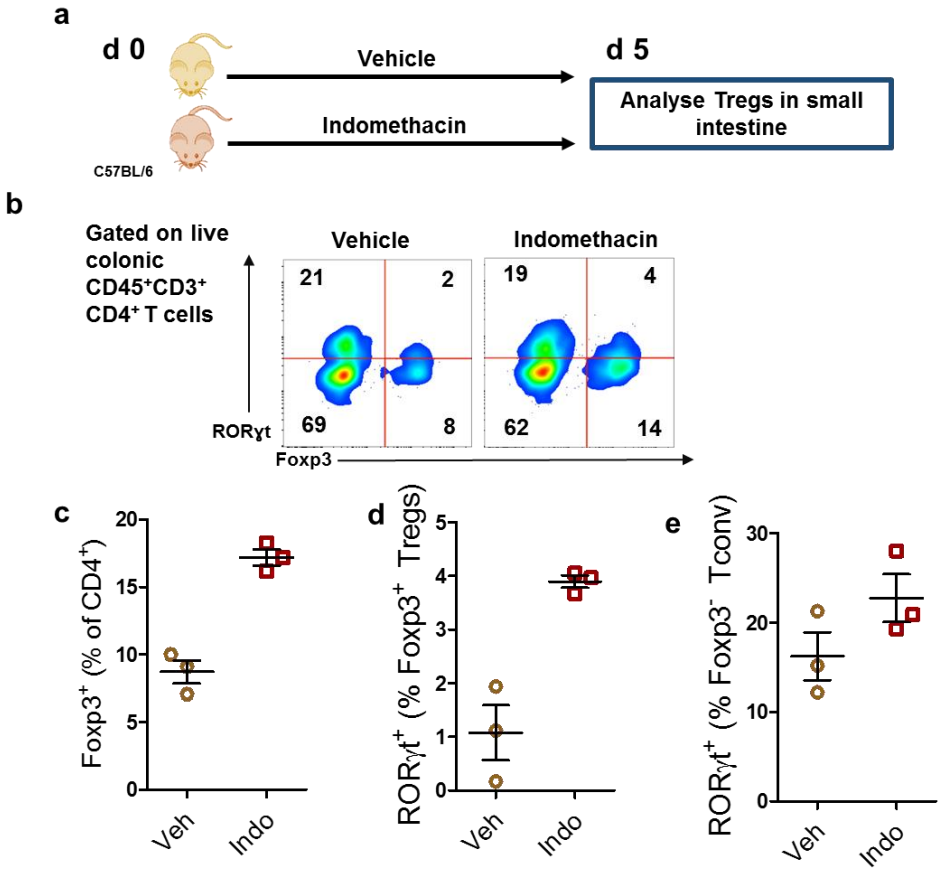


**Figure 4.3: Effect of *in vivo* inhibition of endogenous $PGE_2$ on small intestine RORγt$^+$Foxp3$^+$ Treg production.** *(a) Experimental timeline for in vivo inhibition of endogenous $PGE_2$. WT C57BL/6 mice were treated with either vehicle, or indomethacin in drinking water for 5 days. Mice were culled on d 5, tissues removed and processed. (b) Expression of Foxp3 and RORγt in live CD4$^+$ T cells in the small intestines of mice treated with either vehicle or indomethacin. Representative dot plots were gated on live CD45$^+$CD3$^+$CD4$^+$ cells. (c) Percentages of Foxp3$^+$ cells within total live CD4$^+$ cells in small intestines of mice treated under different conditions. (d) RORγt expression within total live Foxp3$^+$CD4$^+$ cells in the small intestine of mice treated under different conditions. (e) Total proportion of RORγt$^+$ cells within Foxp3$^-$ Tconv cells in the small intestine of mice treated under different conditions. (n = 3). Data shown as means ± SEM (error bars). Data representative of one independent experiments.*

### 4.3.4 Inhibition of $PGE_2$ signalling can result in an increased inflammatory environment within the colon

It is clear that use of NSAIDs, such as indomethacin can increase the proportion of RORɣt$^+$ Tregs within the intestinal environment (**Fig 4.2d, Fig. 4.3c**). Although there was no significant increase in any pro-inflammatory RORɣt$^+$Foxp3$^-$ T conventional cells, it has been thought that long-term NSAID use can exacerbate colonic inflammation [168]. Flow cytometry was used to detect pro-inflammatory cytokines within the colonic tissue.

As before, WT C57BL/6 mice were treated with either vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) for one week, colons extracted and a single cell suspension was formed. Cells were cultured for five hours with ionomycin, Golgi plug and PMA to stimulate cytokine production prior to staining for T cell and inflammatory cytokine markers (**Fig. 4.4a**). Cells were gated on live CD45$^+$CD3$^+$CD4$^+$ T cells, and the gating strategy for the inflammatory markers IL-17 and IFNɣ are demonstrated in **figure 4.4b**. There is a slight increase in the number of IL-17 expressing cells in the colon of indomethacin-treated mice, and this is mirrored by the mean fluorescence intensity (MFI) ($p = 0.07$) (**Fig. 4.4c**). This is similarly observed with both the number ($p = 0.07$) and MFI of IFNɣ expressing cells (**Fig. 4.4d**).

Inhibition of the COX enzymes by NSAIDs affects the arachidonic acid (AA) metabolism pathways [89]. AA is pushed towards leukotriene formation, and these compounds recruit neutrophils to the site of production. Therefore, to see whether use of indomethacin likewise enhanced neutrophil infiltration, CD45$^+$CD3$^-$CD11b$^+$CD11c$^+$ cells were also stained for lymphocyte antigen 6 complex locus G6D (Ly6G), a neutrophil marker, and gating strategy is demonstrated in **figure 4.4e**. As expected, there was a significantly greater proportion ($p \leq 0.01$), and number of neutrophils ($p \leq 0.05$) in the indomethacin-treated mice colons compared to those treated with vehicle (**Fig. 4.4f**).

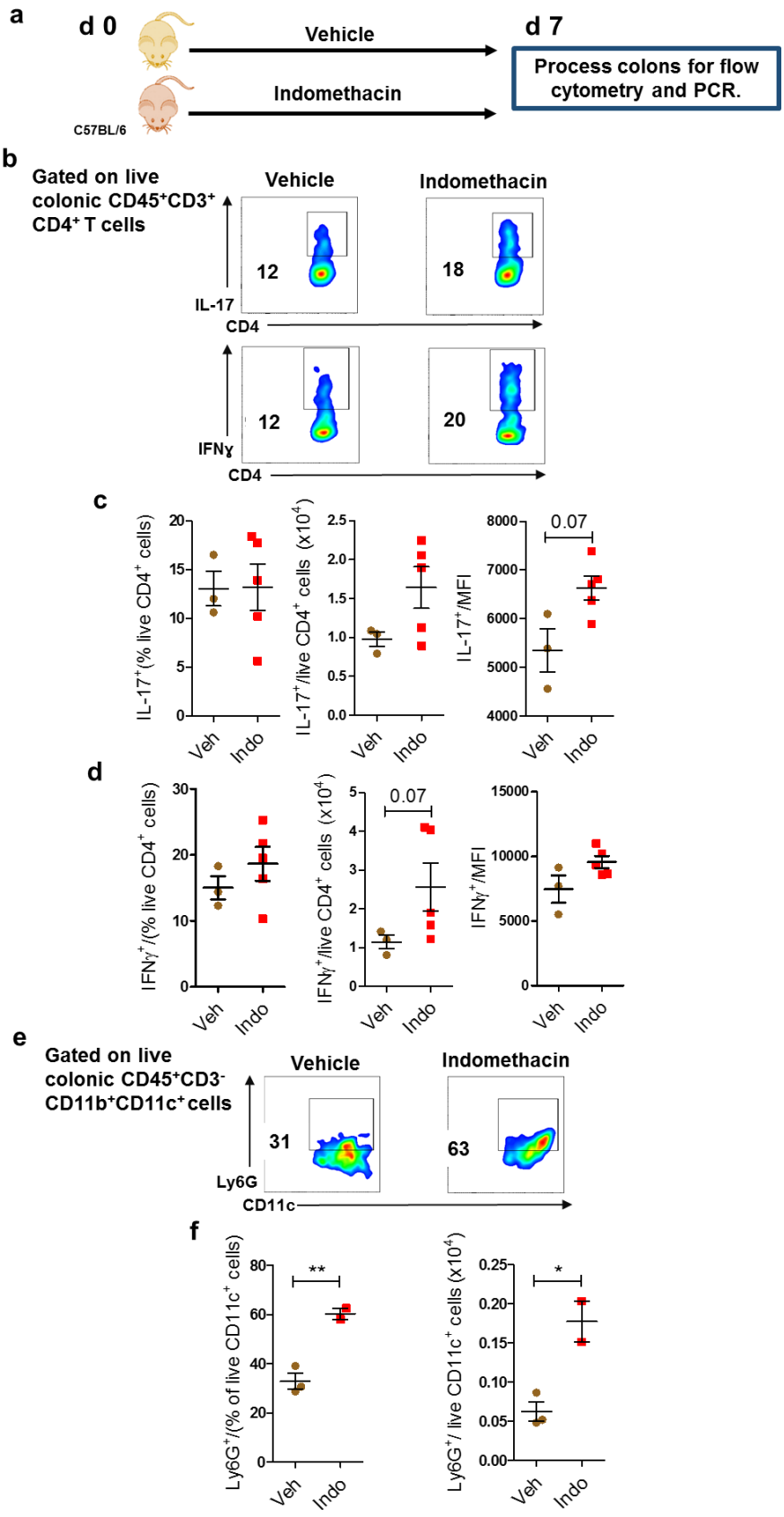
a
d 0
Vehicle
Indomethacin
d 7
Process colons for flow cytometry and PCR.
C57BL/6
b
Gated on live colonic CD45+CD3+ CD4+ T cells
Vehicle
Indomethacin
12
18
IL-17
CD4
12
20
IFNγ
CD4
c
IL-17+(% live CD4+ cells)
IL-17+/live CD4+ cells (x10^4)
IL-17+/MFI
0.07
Veh
Indo
d
IFNγ+/(% live CD4+ cells)
IFNγ+/live CD4+ cells (x10^4)
IFNγ+/MFI
0.07
Veh
Indo
e
Gated on live colonic CD45+CD3- CD11b+CD11c+ cells
Vehicle
Indomethacin
31
63
Ly6G
CD11c
f
Ly6G+/(% of live CD11c+ cells)
Ly6G+/ live CD11c+ cells (x10^4)
**
*
Veh
Indo

**Figure 4.4: The effect on colonic inflammatory markers after inhibition of $PGE_2$.** *(a) Experimental timeline for in vivo inhibition of endogenous $PGE_2$. WT C57BL/6 mice were treated with either vehicle, or indomethacin in drinking water for 7 days. Mice were culled on d 7, colons removed and processed. Cells were incubated for four hours with PMA, ionomycin and Golgi plug, prior to staining of intra-cellular cytokines, or with anti-Ly6G antibody as a neutrophil marker. (b) Flow cytometry plot illustrates the gating strategy for IL-17 and IFNɣ in live $CD4^+$ T cells from colonic tissue. (c) Graphs show percentage, number and MFI of $IL\text{-}17^+$ expressing cells within total live $CD4^+$ cells in colons of mice treated under different conditions. (d) Graphs show percentage, number and MFI of $IFN\gamma^+$ expressing cells within total live $CD4^+$ cells in colons of mice treated under different conditions. (e) Flow cytometry plot illustrates the gating strategy for Ly6G, a neutrophil marker, in live $CD11b^+CD11c^+$ cells within colonic tissue. (f) Graphs show percentage and total number of $Ly6G^+$ cells within total live $CD11b^+CD11c^+$ cells within the colons of mice treated under different conditions. Experiment repeated twice. Data shown as means ± SEM (error bars) (n = 2 - 5) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ by Mann-Whitney U.*

### 4.3.5 Long-term low dose indomethacin treatment does not result in colonic inflammation

It is widely known that use of NSAIDs is associated with negative effects in the lower section of the G.I tract [168]. The adverse effects include; colonic ulceration and reduced mucosal hydrophobicity, which create an ineffective barrier allowing commensal microbiota to potentially cross the epithelial barrier and enter peripheral sites causing inflammation [98, 168, 169]. A lower dose of indomethacin was used to block endogenous $PGE_2$ production over a longer period to see whether this had a similar effect of the higher dose over a short period.

WT C57BL/6 mice were treated with either vehicle (0.25 % ethanol) or indomethacin at 2 mg/kg/day for two or 1 mg/kg/day for four weeks, before colons were extracted and processed for flow cytometry (**Fig. 4.5a**). Flow cytometry dot plot of Foxp3 and RORγt expression in live $CD4^+$ T cells from mice treated with vehicle or indomethacin at 2 mg/kg/day for two weeks is demonstrated in **Figure 4.5b**. There was a significantly greater proportion of $Foxp3^+$ Tregs in colons treated with low dose indomethacin (2 mg/kg/day) for two weeks compared to the vehicle group ($p \leq 0.01$), whereas there was no increase in the pro-inflammatory $ROR\gamma t^+$ Tconv cells (**Fig. 4.5b**). Flow cytometry dot plot of Foxp3 and RORγt expression in live $CD4^+$ T cells from mice treated with vehicle or indomethacin at 1 mg/kg/day for four weeks is demonstrated in **figure 4.5c**. There was also a significantly greater number ($p \leq 0.05$) of $Foxp3^+$ Tregs in colons treated with low dose indomethacin (1 mg/kg/day) for four weeks compared to the vehicle group, whereas there was no increase in the pro-inflammatory $ROR\gamma t^+$ Tconv cells, similar to what was observed with 2 mg/kg/day indomethacin over two weeks (**Fig. 4.5c**). These data demonstrates that low dose of indomethacin still results in increased colonic lamina propria Tregs.

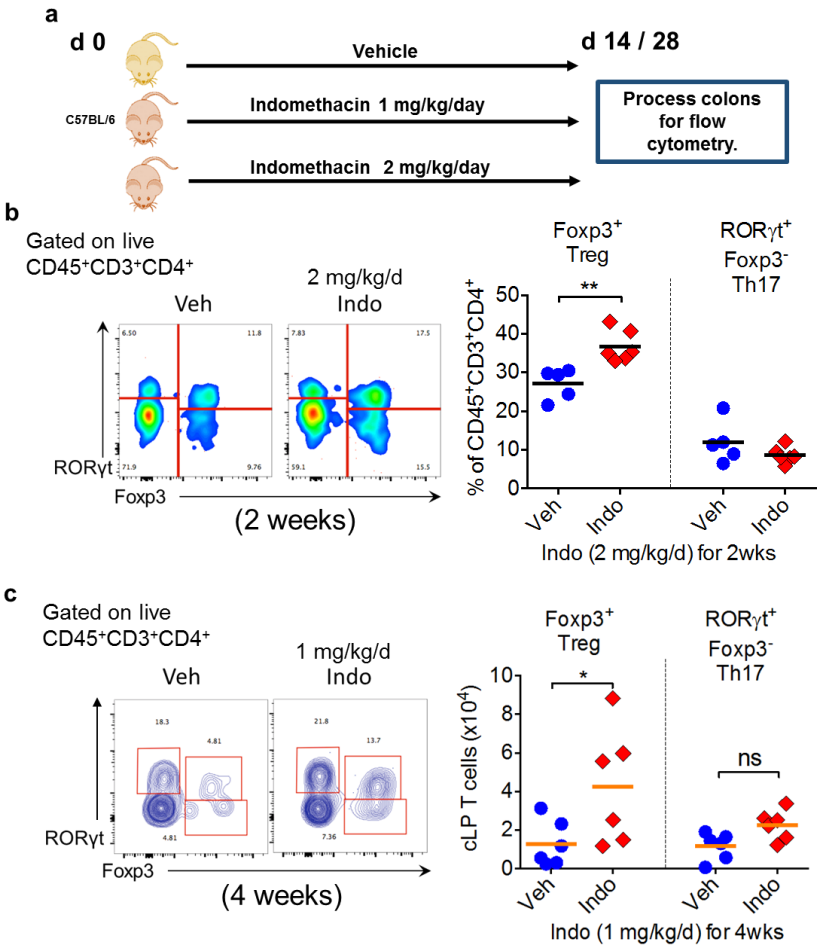


**Figure 4.5: The effect of low dose indomethacin treatment on colonic Tregs.** *(a) Experimental timeline for in vivo inhibition of endogenous $PGE_2$. WT C57BL/6 mice were treated with either vehicle, or indomethacin at 1 mg/kg/day or 2 mg/kg/day in drinking water for 28 days or 14 days respectively. Mice were culled on day 14 or day 28, colons extracted and processed for flow cytometry. (b) Flow cytometry dot plots for RORγt and Foxp3 expression within live $CD45^+CD3^+CD4^+$ T cells in colons from mice treated with 2 mg/kg/day indomethacin or vehicle for two weeks. Proportion of colonic $Foxp3^+$ Tregs and $ROR\gamma t^+$ Tconv cells from mice treated with 2 mg/kg/day indomethacin or vehicle for two weeks. (c) Flow cytometry dot plots for RORγt and Foxp3 expression within live $CD45^+CD3^+CD4^+$ T cells in colons from mice treated with 1 mg/kg/day indomethacin or vehicle for four weeks. Graphs to show number of colonic $Foxp3^+$ Tregs and $ROR\gamma t^+$ Tconv cells from mice treated with 1 mg/kg/day indomethacin or vehicle for four weeks. Data shown as means ± SEM (error bars) (n = 5 - 6) * $p \leq 0.05$, ** $p \leq 0.01$ by Mann-Whitney U.*

### 4.3.6 EP4 signalling in T cells does not affect colonic Tregs at a steady state

Loss-of-function (e.g. through indomethacin use) and gain-of-function (e.g. using the EP4 agonist) studies showed an inhibitory effect of $PGE_2$-EP4 signalling on intestinal Tregs. To further investigate whether $PGE_2$ regulates intestinal Tregs through direct action on T cells, or indirectly via other cells, I used mice with specific deletion of the EP4 receptor in T cells. $LCK^{cre}EP4^{fl/fl}$ mice were used to determine the effect of the EP4 receptor on endogenous Treg expression.

To examine whether the cell populations were different *in vivo* compared to WT C57BL/6 mice under steady-state conditions, $EP4^{fl/fl}$ (control) or $LCK^{cre}EP4^{fl/fl}$ (KO) mice were culled and tissues processed. Flow cytometry dot plots showing CD25, Foxp3 and RORγt expression in $CD4^+$ T cells are shown in **figure 4.6a**. There was a similar proportion and number of $CD25^+Foxp3^+$ cells between the tissues from the EP4 KO and control mice (**Fig. 4.6b**). However inhibition of T cell $PGE_2$ signalling in EP4 KO mice resulted in a greater proportion of RORγt$^+$ Tregs in the colonic lamina propria ($p \leq 0.05$), albeit total numbers were not affected. This suggests that inhibition of RORɣt$^+$ Tregs is only partially due to T cell involvement (**Fig. 4.6b - e**). This was also mirrored by an increase in RORγt MFI in colons of KO mice compared to control mice (**Fig. 4.6f**).

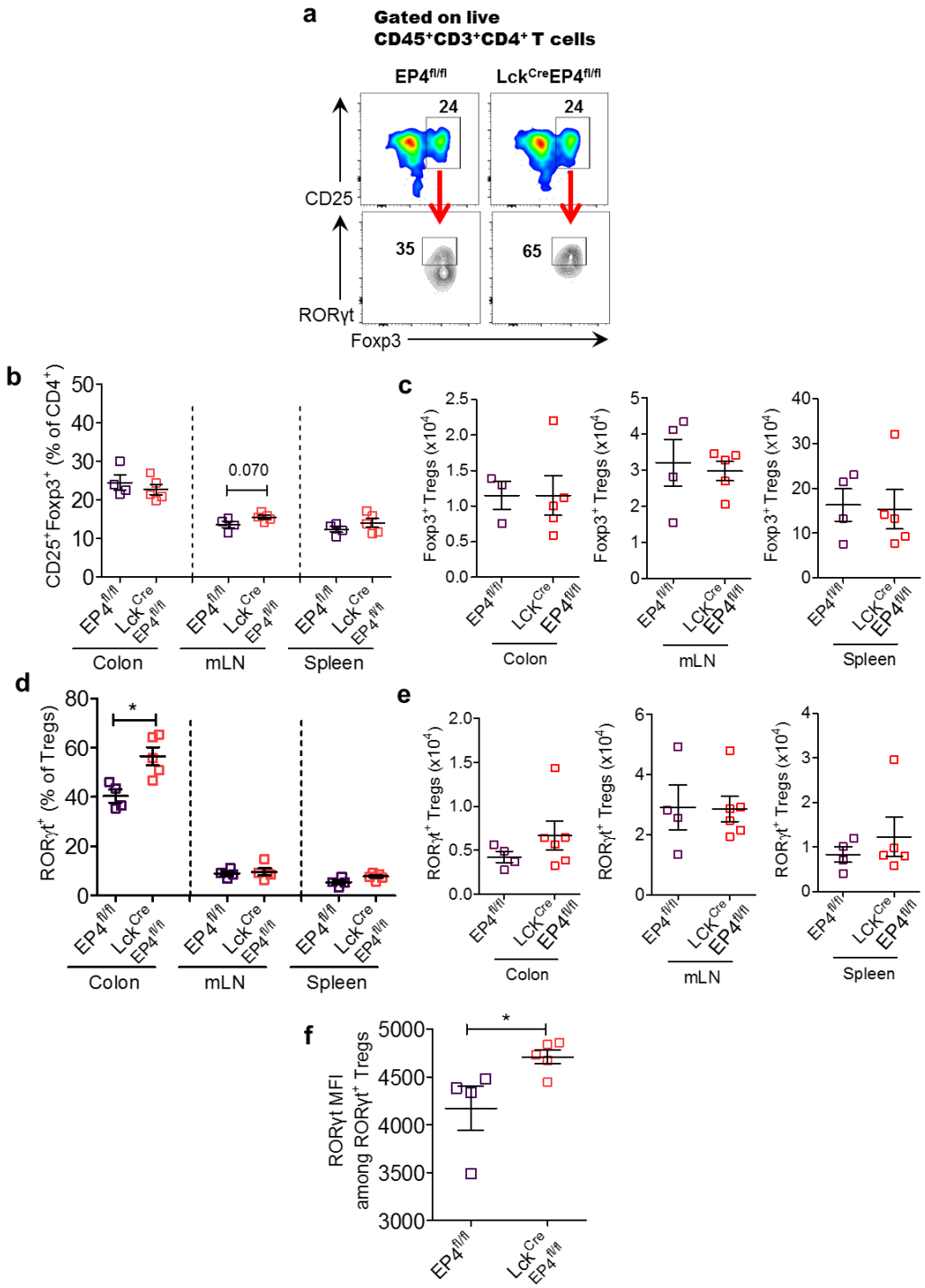
a
Gated on live
CD45+CD3+CD4+ T cells
EP4fl/fl
LckCreEP4fl/fl
24
24
CD25
35
65
RORγt
Foxp3
b
CD25+Foxp3+ (% of CD4+)
0.070
EP4fl/fl
LckCre EP4fl/fl
Colon
mLN
Spleen
c
Foxp3+ Tregs (x10^4)
Colon
mLN
Spleen
d
RORγt+ (% of Tregs)
*
Colon
mLN
Spleen
e
RORγt+ Tregs (x10^4)
Colon
mLN
Spleen
f
RORγt MFI
among RORγt+ Tregs
*
EP4fl/fl
LckCre EP4fl/fl

**Figure 4.6: Effect of EP4 signalling in T cells at a steady state**. *(a) Expression of RORγt and Foxp3 in CD25$^+$ T cells from the colons of naïve mice at a steady state. Representative dot plots were gated on live CD45$^+$CD3$^+$CD4$^+$ cells. (b) Graphs show percentages of Foxp3$^+$ T cells within live CD4$^+$ T cells in spleens, mLNs and colons of EP4 KO or control mice. (c) Graphs show number of Foxp3$^+$ T cells within live CD4$^+$ T cells in spleens, mLNs and colons of EP4 KO or control mice. (d) Graphs show percentages of RORγt$^+$Foxp3$^+$ Tregs within live CD4$^+$ T cells in spleens, mLNs and colons of EP4 KO or control mice. (e) Graphs show number of RORγt$^+$Foxp3$^+$ Tregs within live CD4$^+$ T cells in spleens, mLNs and colons of EP4 KO or WT mice. (f) Graphs show RORγt$^+$ MFI among RORγt$^+$ Tregs. Data shown as means ± SEM (error bars) are pooled from two independent experiments. (n = 4 - 6) * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 by Mann-Whitney U test.*

## 4.4 Discussion

Data presented in this chapter suggest that $PGE_2$ suppresses intestinal $Foxp3^+$ Tregs, especially the $ROR\gamma t^+Foxp3^+$ Tregs, through its receptor EP4 which is consistent with my previous *in vitro* work.

The *in vivo* role of $PGE_2$ on tissue resident Tregs was determined by treating mice with a vehicle, indomethacin to inhibit $PGE_2$ production, or indomethacin plus an EP4 agonist to specifically activate this pathway. Addition of the EP4 agonist to specifically activate this $PGE_2$ signalling pathway prevented an increase in $Foxp3^+$ Tregs within each tissue that had been observed with mice treated with indomethacin only (**Fig. 4.1**). This difference was detected most significantly within the colonic tissue, which is why the subsequent work focused on this organ. Within this environment, there are a subtype of $Foxp3^+$ Tregs that express RORγt (40 – 60 %) that are vital in suppressing pro-inflammatory cells, therefore this marker was measured by flow cytometry to determine whether this subtype of Tregs were similarly influenced by inhibition of endogenous $PGE_2$. As expected, it was only within the colonic environment that the proportion of $ROR\gamma t^+Foxp3^+$ Tregs were affected by indomethacin treatment (**Fig. 4.2**). There was also a greater number of these cells detected in the mLNs of indomethacin treated mice (no significant difference in percentage) that was not observed in the spleen. This suggests that there is increased migration of these highly suppressive $ROR\gamma t^+$ Tregs from the colon to the mLNs (**Fig 4.2**).

The increase of $ROR\gamma t^+$ Tregs was likewise observed with the SI (**Fig. 4.3**). The SI and colon are similar organs in that they are both key components of the gastrointestinal tract and experience a variety of food antigens from diet and commensal microbiota along its surface, however they are also greatly different due to the pH gradient that is present along the tract. The colon is a less acidic environment compared to the SI and therefore has a greater commensal diversity which in turn can influence immune cells within the environment as previously discussed [170]. This suggests there are similar factors between the colon and SI that are being influenced by inhibition of endogenous $PGE_2$.

Initially it was thought that intestinal Tregs may have been enhanced after indomethacin treatment due to inflammation, evidenced by slight increases in inflammatory cytokines (IFN-γ, IL-17), compared to the vehicle group (**Fig. 4.4**) [168]. Duffin's (2016) finding which showed mice with reduced $PGE_2$ synthesis developed systemic inflammation associated with gut microbiota infiltration, however they showed this to be due to reduced ILC3 proliferation and IL-22 production consequently affecting the gut barrier integrity [112]. NSAIDs are commonly used for treatment of inflammatory diseases, however they have been shown to be ineffective at treating patients with systemic inflammation due to damage to intestinal barriers and bacterial infiltration, worsening symptoms [112]. In this model of $PGE_2$ inhibition there was no increase in T conventional cells in the colons of indomethacin treated mice, albeit a slightly greater proportion of inflammatory cytokines, such as IL-17 (**Fig. 4.1, 4.4**).

RORγt$^+$ Tregs are vitally important within the colonic environment to suppress inflammation, and have a greater flexibility to respond to signals due to the homing molecules CD62L and mTGFβ [167]. It has been demonstrated that RORγt$^+$Foxp3$^+$ Tregs are better at protecting against colitis induction compared to RORγt$^-$Foxp3$^+$ Tregs, which demonstrates the importance of understanding the mechanism for induction [55].

To ensure the increase in Tregs was not due to initiation of an inflammatory phenotype within the colonic environment, mice were treated with a lower dose of indomethacin 1 mg/kg/day for 4 weeks or 2 mg/kg/day for 2 weeks, prior to measuring the levels of RORγt and Foxp3 expression in colonic cells. At both 2 and 4 weeks, vehicle-treated mice had a significantly reduced number of Foxp3$^+$ Tregs compared to mice treated with the low dose indomethacin (**Fig. 4.5**). Whereas there was no increase in Tconv cells, or RORγt$^+$ Tconv cells (**Fig. 4.5**) suggesting that the increase in RORγt$^+$ Tregs was not due to the inflammation.

It was shown in **figure 4.2** that stimulation of EP4, the key $PGE_2$ receptor, prevented an increase of colonic RORγt$^+$Foxp3$^+$ Tregs in indomethacin treated mice. To further confirm the importance of this receptor, Tregs were measured in T cell specific EP4 KO mice under steady-state conditions. Contrary to expectations, there was no difference in Foxp3$^+$ Tregs within the tissues (**Fig. 4.6**), however there was a significantly greater proportion of RORɣt$^+$ Tregs in the colon (**Fig. 4.6**), demonstrating that inhibition of Tregs was only partially due to T cell involvement. Additionally, both EP4 KO and WT mice will have a similar level of basal nTregs present in the colon which may be why similar levels of Foxp3 were detected, whereas colonic pTreg differentiation will be affected in the EP4 KO mice, and as mentioned before, approximately 40 – 60 % of this population co-express RORγt [55].

T cell specific EP4 KO mice only saw an increase in colonic RORγt$^+$ Tregs, Foxp3 levels were unaffected by disrupting $PGE_2$ signalling in T cells, contradictory to what was observed in the *in vitro* results (**chapter 1, section 3.3.1 – 3.3.4).** These findings suggested that there are other factors involved in $PGE_2$'s suppression of colonic Tregs *in vivo*.

Previous work has demonstrated that an increased commensal microbiota correlates with augmented colonic Tregs, and also the number of antibiotics prescribed during childhood is associated with an escalated risk of IBD [51, 55, 171]. Since the microbiome has been shown to be so important to the induction and regulation of the immune system, it was then thought that possibly the microbiota were involved in $PGE_2$'s suppression of colonic Tregs. In the following chapter, antibiotics were used to deplete gut microbiota, to determine whether these were involved in $PGE_2$'s modulation of colonic Tregs, and specific genetically modified mice were used to determine the pathways involved in this.

## 4.5 Conclusion

It has been demonstrated that the $PGE_2$-EP4 T cell signalling pathway was only partially involved in suppressing colonic lamina propria Foxp3$^+$ Tregs. Therefore, the next questions that were asked were; are commensal microbiota involved in $PGE_2$'s suppression of colonic Tregs, and are there any other cell types involved?

# 5 Mechanisms for $PGE_2$ Suppression of Intestinal Tregs

## 5.1 Introduction

In the previous chapter it was demonstrated that inhibition of endogenous $PGE_2$ enhanced colonic $Foxp3^+$ Tregs, especially the $ROR\gamma t^+Foxp3^+$ Tregs. In this chapter, the mechanism for $ROR\gamma t^+$ Treg induction within this environment was then examined.

The human gut contains more than 100 trillion microbes, of which a large number play an important role in disease and health [172]. Microbiota can breakdown indigestible carbohydrates providing energy for both itself and the host. The resulting products such as short-chain fatty acids (SCFAs) can benefit the immune system through induction of Tregs [49, 172]. These products of a fibrous diet can lower the intestinal pH and consequently influence the population of gut microbiota which inhabit this environment [57]. SCFAs such as butyrate, boost pTreg differentiation within the gut, however they have no effect on tTregs. The importance of gut microbiota in influencing intestinal products, and also their influence on the intestinal immune response was shown by Smith. P, *et al* (2013) [70]. They demonstrated that germ-free (GF) mice had a reduced concentration of important SCFAs such as butyrate, and lower number of colonic Tregs compared to wild type (WT) mice [70]. Tregs are required to prevent excessive expansion of Th cells due to signals via the microbiota and also enables survival [172]. Intestinal Tregs (and dendritic cells (DCs)) have the highest expression of the SCFA receptor GPR43 compared to those in the spleen and mesenteric lymph nodes (mLNs) which demonstrates the importance of microbial products on intestinal immune cell development [70]. SCFAs signal via this receptor to stabilise Treg Foxp3 expression through acting as a histone deacetylase (HDAC) inhibitor, and boosting Treg function through increased IL-10 expression and suppressive ability [70].

The gut microbiota influences intestinal Treg development, for example *Clostridia* can colonise the mucosal layer, and exert influence on the intraepithelial cells to produce TGFβ and Indoleamine 2,3-dioxygenase (IDO), both which are important in conversion and activation of Tregs respectively [59].

The gut microbiota influences intestinal Treg development, therefore it was questioned whether the intestinal DC subsets, which convert naïve T cells into their various subsets partially through processing bacterial peptides, were also affected by suppression of $PGE_2$. Ohnmacht. C, *et al* (2015) demonstrated that not only do RORγt$^+$ Tregs require gut microbiota for development, but they are also dependent on MHCII$^+$ DCs [167]. DCs process proteins and display antigens on the surface, and depending on whether they have encountered a pro- or anti-inflammatory stimulus at the site of conversion, this determines the cytokines produced which influences differentiation of naïve T cells into either effector or regulatory T cells. Feng. T, *et al* (2010) showed *in vitro* that MyD88 knockout (KO) DCs induced less FOXP3 expression in naïve T cells, compared to WT DCs indicating that signalling via this receptor is important for Treg induction and optimal TGF-β production [173]. BMDCs express aldehyde dehydrogenases (ALDH)1 and ALDH2, and provide retinoic acid to DC precursors in the intestinal environment [173]. Retinoic acid can enhance expression of the gut homing molecule CCR9 on both DCs and Tregs.

CD103$^+$ DCs are thought to be vital in generating Tregs via TGF-β and retinoic acid, a vitamin A metabolite but also critical for mucosal Th17 cell differentiation [6, 68, 69]. Likewise, CD103$^-$ DCs have recently been shown to be important in generating Tregs [6, 37]. CD103$^-$ DCs are able to maintain intestinal homeostasis in mechanisms which involves the gut microbiota, while CD103$^+$ DCs acts independently of the microbiome [6, 37]. It was demonstrated that CD11b$^+$CD103$^-$ DCs produced IFNβ after stimulation by gut microbiota, and subsequently enhanced proliferation of Foxp3$^+$ Tregs [6].

IFNβ is already used as a vital treatment for Multiple Sclerosis (MS) [174, 175]. IFNβ treatment not only increases the production of Tregs to suppress the pro-inflammatory response, but also reduces T cell IL-6R expression, and subsequently enables effector T cells to be more responsive to the suppressive activity of Tregs [174, 175]. The aetiology of MS is still not known; however, it is likely to be a combination of environmental and genetic factors. It has also been suggested that MS could be triggered by dysbiosis of commensal microbiota [175].

Berer, K. *et al* (2011) used mice genetically altered to express myelin basic protein (MBP) to demonstrate that commensal microbiota were required for the development of spontaneous experimental autoimmune encephalomyelitis (EAE), an animal model of human MS [175, 176]. Germ-free mice were protected from spontaneous EAE induction, whereas specific-pathogen-free mice did develop disease highlighting the involvement of gut microbiota in the immune system [175, 176].

After production of IFNβ, it binds to the IFN receptor resulting in phosphorylation of STAT-1 and STAT-2 by JAK and TYK2 [167]. The consequent phosphorylated products form a complex with IRF7 to translocate into the nucleus and bind to the interferon-stimulated response element (ISRE) to further enhance transcription of interferon-related genes [167]. IFNβ is important in maintaining Foxp3 expression, Treg suppressive function and has been shown more recently to enhance Treg proliferation [74, 167]. Lee, E. E, *et al* (2012) demonstrated the importance of interferon signalling using a T cell colitis disease model [74]. RAG mice had worse symptoms when IFNAR KO Tregs were injected compared to the mice injected with WT Tregs [74].

It was described in **chapter 3 and 4** that *in vivo* inhibition of $PGE_2$ increased colonic $ROR\gamma t^+Foxp3^+$ Tregs, and this was prevented by addition of an EP4 agonist. In T cell specific EP4 knock-out mice, there was no difference in Foxp3 expression, however there was significantly greater RORγt expression, suggesting that $PGE_2$ signalling via T cell EP4 receptors were only partially involved in suppression of $ROR\gamma t^+$ Tregs. The effect of $PGE_2$ on colonic Treg development was of most interest after initial experiments, hence broad-spectrum antibiotics were also utilised later to deplete intestinal microbiota, to determine whether these were involved in $PGE_2$'s modulation of intestinal Tregs.

## 5.2 Experimental methodology

To determine the involvement of gut microbiota in $PGE_2$'s modulation of colonic Treg number, broad-spectrum antibiotics (details in **chapter 2, section 2.8.2**) were used to deplete intestinal microbiota. The combination and concentration of broad-spectrum of antibiotics have been previously used by multiple groups to deplete the gut microbiota [112, 151, 177]. The importance of the gut microbiota was aslso confirmed by the use of MyD88/TRIF double knockout mice, which are deficient in the downstream TLR adapter molecules MyD88 and TRIF, which would prevent gut microbial signalling via the immune cells.

Another important subset of immune cells that are involved in naïve T cell differentiation are colonic lamina propria mononuclear phagocytes (cLP MNPs). cLP MNPs are important for processing microbial peptides and signalling for naïve T cells to be converted to either pro- or anti-inflammatory T cells, therefore these were examined to see whether inhibiting $PGE_2$ affected the cLP MNP subsets activated within colon tissue. As before, mice were treated with vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) to block endogenous $PGE_2$, plus or minus broad-spectrum antibiotics to deplete commensal microbiota, then colons were removed and stained for markers for measuring on the flow cytometer.

To also confirm the involvement of the gut microbiota, MyD88/TRIF DKO mice were treated with vehicle or indomethacin, and colons removed for staining for cLP MNP markers, then measured by flow cytometry. Different MNP subsets have been described as having varied abilities to transform naïve T cells into either a more effector or regulatory cell type, therefore subsets of MNPs will be sorted from mLNs and spleens of WT mice, and co-cultured with naïve T cells sorted from spleens of Foxp3-YFP mice, to confirm previous work that has shown MNP subsets influencing naïve T cell differentiation, and to see if the subset that is increased in colons of indomethacin treated mice have any influence on naïve T cell differentiation *in vitro*.

As previously discussed, recently Nakahashi-Oda. C, *et al* (2016) demonstrated that $CD103^{-}CD11b^{+}$ DCs were able to express IFNβ which enabled colonic Treg proliferation [6]. Therefore, using PCR, type 1 interferon genes were measured in $CD103^{-}CD11b^{+}$ MNPs from both vehicle- and indomethacin-treated mice. Phosphorylated STAT-1 was detected within Treg and MNP cells using flow cytometry as another method to measure the involvement of interferon signalling. In addition to this, IFNAR mice were also treated with vehicle or indomethacin, to confirm the involvement of interferon signalling in colonic Treg and MNP differentiation.

As commensal microbiota were shown to mediate $PGE_2$'s suppression of Tregs *in vivo*, caecum content from vehicle or indomethacin treated mice was extracted and sterilely filtered so only metabolites remained, to see whether metabolites also affected naïve T cell conversion *in vitro*. This solution was added to a naïve T cells – DC co-culture to see whether the metabolites had been altered between the two different treated groups, and whether this would alter Treg differentiation.

### 5.2.1 Aims of chapter

- To determine the involvement of gut microbiota in $PGE_2$'s suppression in intestinal Tregs.
- To determine the involvement of DCs in intestinal Treg induction.
- To define the mechanism for $PGE_2$'s suppression of intestinal Tregs.

## 5.3 Results

### 5.3.1 Gut microbiota mediate $PGE_2$ suppression of colonic Tregs

Inhibition of colonic RORγt$^+$ Tregs by $PGE_2$ was only partially due to $PGE_2$-EP4 signalling in T cells, suggesting the involvement of other mechanisms through $PGE_2$'s indirect action on T cells. Gut microbiota are important for colonic RORγt$^+$ Tregs development, as germ-free (GF) mice have significantly fewer colonic RORγt$^+$ Tregs compared to WT mice, and levels were restored after recolonization by the microbes which demonstrate their importance [55].

To determine whether microbiota were involved in the inhibition of RORγt$^+$ Tregs by $PGE_2$, WT mice were treated with broad-spectrum antibiotics (**chapter 2, table 4**) for two weeks to deplete commensal bacteria and indomethacin (5 mg/kg/day) or vehicle (0.5 % ethanol) for the second week. On day 14, mice were culled, tissues removed and cells stained for Treg markers which were detected by flow cytometric analysis (**Fig. 5.1a**). Flow cytometry dot plots for Foxp3 and RORγt expression in live CD4$^+$ cells are demonstrated in **Fig. 5.1b**. As observed in **chapter 4,** inhibition of $PGE_2$ resulted in a significantly greater proportion ($p \leq 0.01$) and number of colonic Foxp3$^+$ Tregs ($p < 0.01$) compared to the vehicle group. Whereas co-treatment with broad-spectrum antibiotics prevented the increase of Foxp3$^+$ Tregs ($p \leq 0.01$) (**Fig. 5.1c**). This was similarly observed with the proportion and number of RORγt$^+$ expressing colonic Tregs, in which co-treatment of antibiotics resulted in levels analogous to the vehicle-treated group (**Fig. 5.1d**). This indicates that $PGE_2$ is influencing the gut commensal bacteria which consequently affects colonic Treg development.

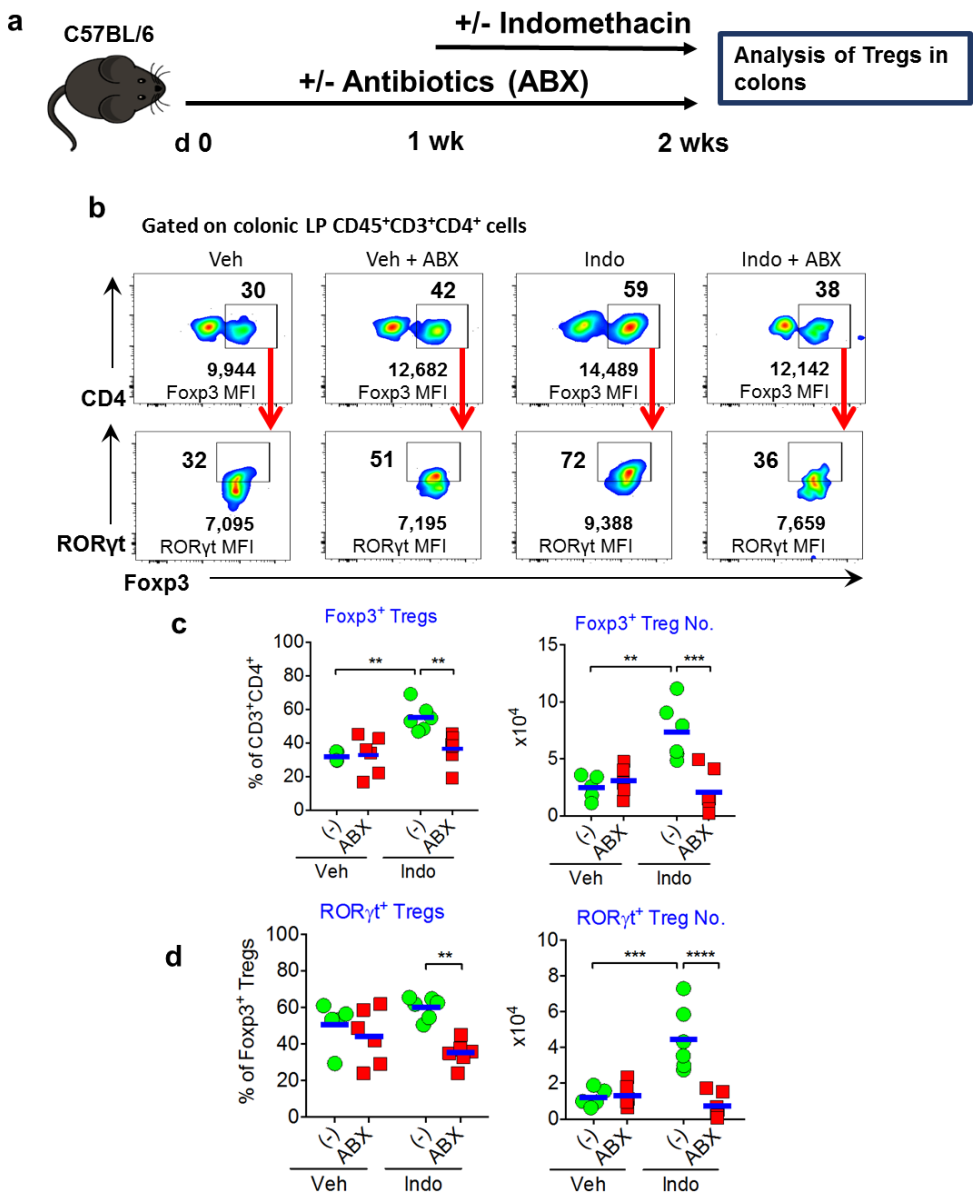


**Figure 5.1: The involvement of gut microbiota in $PGE_2$ regulation of intestinal Tregs**. *(a) Experimental timeline for depletion of gut microbiota and in vivo inhibition of endogenous $PGE_2$. WT C57BL/6 mice were treated with either vehicle, or broad-spectrum antibiotics for seven days before also given either vehicle or indomethacin for a further week in drinking water. Mice were culled on d 14, tissues removed and processed. Cells were stained with anti-Foxp3 and anti-RORγt antibody. (b) Flow cytometry plot of Foxp3 and RORγt expression in live $CD4^+$ T cells from colonic tissue. (c) Total number and proportion of $Foxp3^+$ cells within total live $CD4^+$ cells in colons of mice treated under four different conditions. (d) Total number and proportion of $ROR\gamma t^+$ cells within total live $Foxp3^+$ Tregs cells in colons of mice treated under four different conditions. Data shown as means ± SEM (error bars) are pooled from two independent experiments. (n = 5 - 7) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ by Kruskal-Wallis test, Dunn's Multiple Comparison test used for post-hoc comparison.*

### 5.3.2 Inhibition of endogenous $PGE_2$ is unable to influence colonic RORγt$^+$ Tregs due to TLR adapter molecule knock-out

It was clear from **figure 5.1**, that the gut microbiota are significantly involved in $PGE_2$'s suppression of colonic RORγt$^+$ Tregs. As discussed in **chapter 1**, the intestine is in constant contact with potentially pro-inflammatory products such as; microbiota, viruses, or food antigens. The gut microbiota influences the intestinal immune system via production of microbial metabolites which can be detected by toll-like receptors (TLRs) present on a variety of cells. This prevents an over-reactive immune response during steady-state conditions, maintaining intestinal homeostasis [178]. TLRs, alongside inflammatory markers known as pathogen associated molecular patterns (PAMPs), such as bacterial lipopolysaccharides (LPS), are vital to induce DC migration to peripheral lymphoid organs to either prime effector T cells, or regulatory T cells if there is an absence of pro-inflammatory signals to induce tolerance. Follicle-associated epithelial cells within the intestine aid transmission of bacterial metabolites to intraepithelial immune cells such as DCs, which can then present the peptide to lymphocytes [178]. MyD88 and TRIF are signal adapter molecules required to transmit signals from TLRs through to induction of nuclear factor-kappa-beta (NFκβ), which can influence gene expression, such as the regulatory cytokine IL-10, important in a hypo-responsive situation [179].

To confirm that inhibition of endogenous $PGE_2$ was affecting microbiota and their metabolites, which consequently affected intestinal RORγt$^+$ Treg development, MyD88-TRIF double knockout (DKO) mice were used to determine the effect of inhibiting microbiota signal transduction within cells. MyD88-TRIF DKO mice or WT C57BL/6 mice were treated for five days with vehicle (0.5 % ethanol) or indomethacin (5 mg/ kg/day) in drinking water. On day 5, mice were culled, colons collected and tissues processed for flow cytometry (**Fig. 5.2a**). Dot plots for CD25, Foxp3 and RORγt expression in CD4$^+$ T cells, from either WT or MyD88/TRIF double knockout mice treated with either vehicle or indomethacin, is demonstrated in **figure 5.2b**. As expected, indomethacin treated WT mice had a greater proportion of colonic CD25$^+$Foxp3$^+$ Tregs ($p \leq 0.05$) and RORγt$^+$Foxp3$^+$ Tregs ($p \leq 0.01$) compared to the vehicle WT mice, however there was no difference observed between the vehicle or indomethacin-treated MyD88-TRIF DKO mice Foxp3$^+$ or RORγt$^+$ Treg cell population (**Fig. 5.2c**). This confirms the involvement of gut microbiota in $PGE_2$'s suppression of intestinal Treg development.

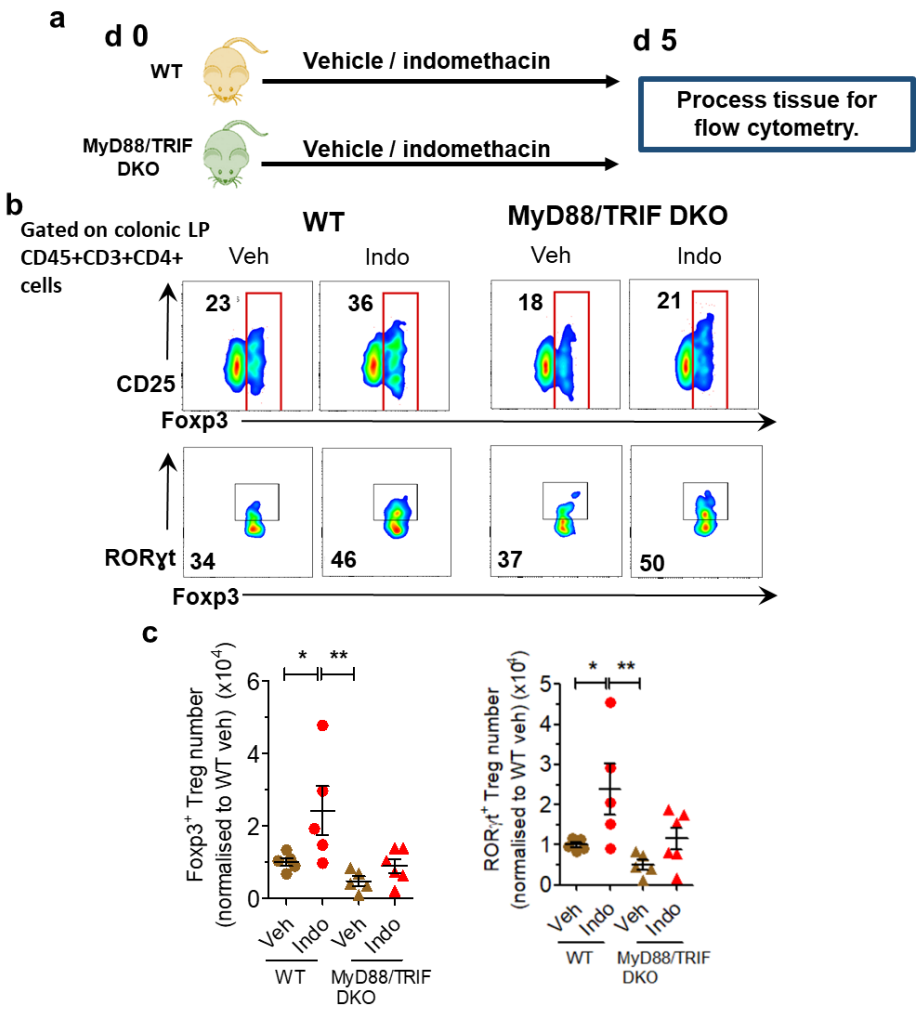


**Figure 5.2: The involvement of toll-like receptor signalling in $PGE_2$ regulation of intestinal Tregs.** *(a) Experimental timeline for in vivo inhibition of endogenous PGE2. MyD88/TRIF DKO or WT C57Bl/6 mice were treated with either vehicle, or indomethacin in drinking water for 5 days. Mice were culled on d 5, colons removed and processed. Cells were stained with Treg antibodies. (b) Flow cytometry gating strategy for Foxp3$^+$ and RORɣt$^+$ Tregs within live colonic CD45$^+$CD3$^+$CD4$^+$ T cells. (c) Graphs show number of Foxp3$^+$ and RORɣt$^+$ Tregs within colons of treated mice normalised to the WT vehicle mice. Data shown as means ± SEM (error bars) are pooled from two independent experiments. (n = 5 – 6) *p ≤ 0.05, **p ≤ 0.01 by Kruskal-Wallis test, Dunn's Multiple Comparison test used for post-hoc comparison.*

### 5.3.3 $PGE_2$ regulates $CD103^-CD11b^+$ cLP MNPs via gut microbiota

There are multiple mechanisms for Treg induction within the gut, as described in more detail in **chapter 1**. For example, there are direct approaches due to SCFA production by gut microbiota that can directly act on the SCFA receptor GPR109a, which both enhances proliferation of these Tregs and also stabilises gene expression [70]. Indirect methods are via gut metabolites activating DCs, and inducing Treg differentiation through retinoic acid and TGF-β production [70]. As MNPs are vital in processing these microbial peptides, and in **Fig. 5.2**, it was observed that MyD88/TRIF DKO mice did not have an increase in $ROR\gamma t^+$ Tregs when treated with indomethacin, the different MNPs subsets were measured to examine whether they were also influenced by the inhibition of $PGE_2$.

Mice were treated as previously stated in **5.3.1**, then colons were processed and stained for cLP MNP markers (**Fig. 5.3a**). The gating strategy for cLP MNP markers is demonstrated in **figure 5.3b**. There was a significantly greater number of colonic $CD103^-CD11b^+$ cLP MNPs in the indomethacin-treated compared to mice treated with vehicle only ($p \leq 0.001$) (**Fig. 5.3c, d**). Co-treatment with broad-spectrum antibiotics prevented the increase in $CD103^-CD11b^+$ MNPs (**Fig. 5.3c, d**). The $CD103^+$ and $CD103^-CD11b^-$ MNPs did not appear to be affected by indomethacin or antibiotic treatment (**Fig. 5.3c, d**). These data suggests that $PGE_2$ negatively affects colonic $CD103^-CD11b^+$ MNP accumulation through modulation of the microbiota which may consequently impact colonic Treg induction.

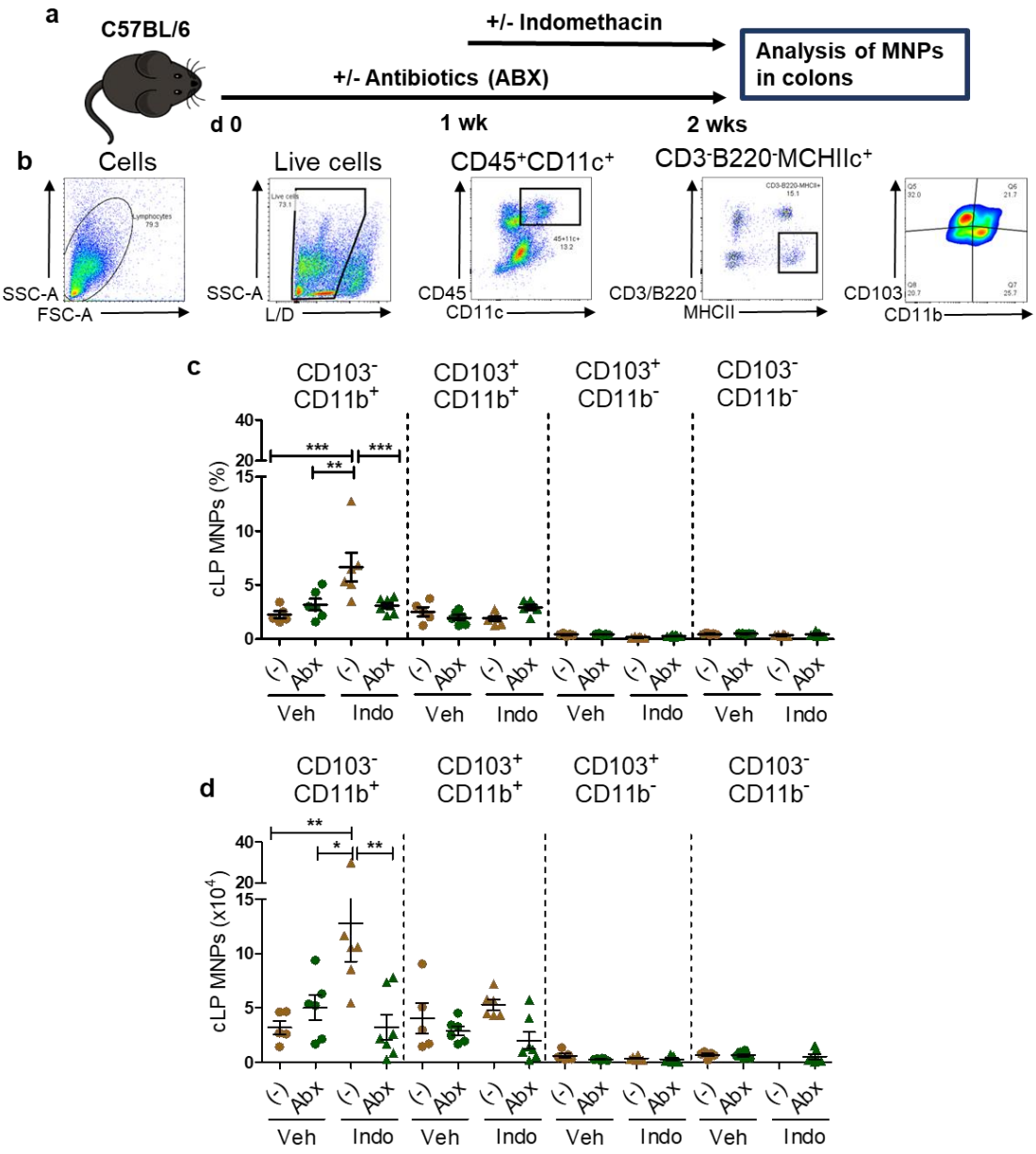


**Figure 5.3: The involvement of gut microbiota in $PGE_2$ regulation of intestinal MPNs.** *(a) Experimental timeline for depletion of gut microbiota and in vivo inhibition of endogenous $PGE_2$. WT C57BL/6 mice were treated with either vehicle, or broad-spectrum antibiotics for seven days before also given either vehicle or indomethacin for a further week in drinking water. Mice were culled on d 14, tissues removed and processed. (b) Flow cytometry plots illustrates the gating strategy for $CD103^+$ or $CD103^-$ MNPs. (c, d) Total number and percentage of $CD103^+$ and $CD103^-$ MNPs within live colonic $CD45^+CD3^-B220^-CD11c^+MHCII^+CD11b^+$ cells. Data shown as means ± SEM (error bars) are pooled from two independent experiments. (n = 5 - 7) * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 by Kruskal-Wallis test, Dunn's Multiple Comparison test used for post-hoc comparison.*

### 5.3.4 Inhibition of endogenous $PGE_2$ is unable to influence $CD103^-$ cLP MNPs due to MyD88/TRIF DKO

$PGE_2$ has been shown to influence colonic $CD103^-CD11b^+$ MNPs via the microbiota (**Fig. 5.3c, d),** and this was still observed after inhibiting $PGE_2$ using low dose indomethacin over a longer period (data not shown), hence the population increase was thought not to be due to inflammation. To confirm the involvement of the microbiota in influencing the $CD103^-CD11b^+$ MNP subpopulation, MyD88/TRIF DKO mice were used to see the effect of inhibiting microbiota signal transduction within cells.

As previously described in **section 5.3.2,** WT or MyD88/TRIF DKO mice were treated with vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) for four days, culled and colons extracted. Tissues were processed and cLP MNPs were analysed by flow cytometry (**Fig. 5.4a**). As expected, indomethacin-treated WT mice had a significantly greater proportion of $CD103^-CD11b^+$ MNPs compared to vehicle-treated WT mice ($p \leq 0.01$) (**Fig. 5.4b, c**). It was also significantly greater than the $CD103^-CD11b^+$ MNPs population from the indomethacin-treated DKO mice ($p \leq 0.05$) (**Fig. 5.4b, c**). Indomethacin and antibiotic treatment had no effect on $CD103^+$ and $CD103^-CD11b^-$ MNPs in both WT and DKO mice. These results support the earlier findings indicating that $PGE_2$ alters microbial metabolite production, hence inhibition of signal transduction negates their ability to influence $CD103^-CD11b^-$ MNPs development.

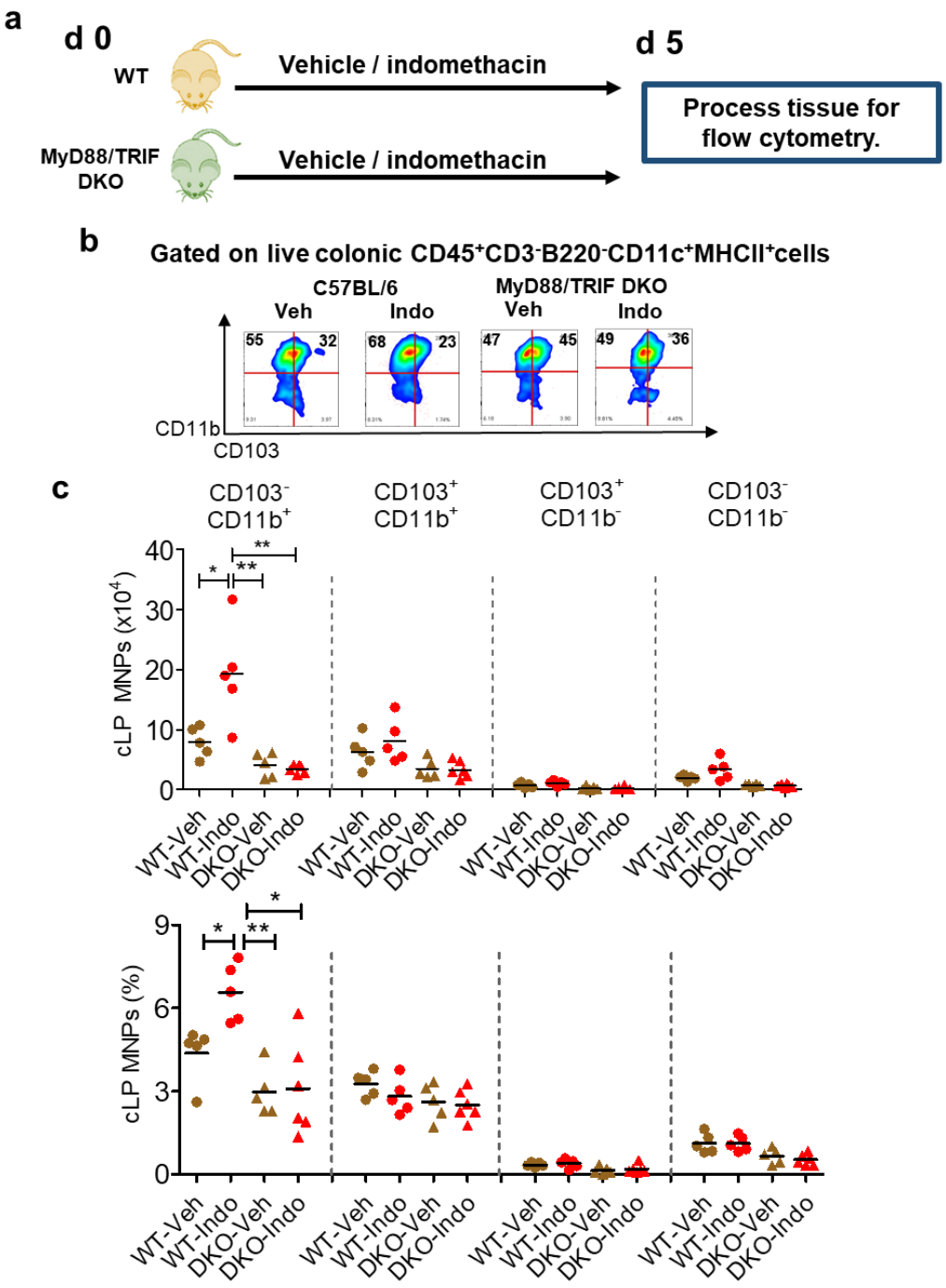


**Figure 5.4: The involvement of TLR signalling in $PGE_2$ regulation of intestinal MNPs.** *(a) Flow cytometry gating strategy for $CD103^-$ or $CD103^+$ MNPs within live colonic $CD45^+CD3^-B220^-CD11c^+MHCII^+$ cells. (b) Graphs show number of $CD103^-$ or $CD103^+$ $CD11b^-$ or $CD11b^-$ MNPs within colons of MyD88/TRIF DKO and WT mice. Data shown as means ± SEM (error bars) are pooled from two independent experiments. (n=5-6) *p ≤ 0.05, **p ≤ 0.01 by Kruskal-Wallis test, Dunn's test used for post-hoc test.*

### 5.3.5 cLP CD103$^-$ MNPs enhance RORγt$^+$Foxp3$^+$ Tregs *in vitro*

It was observed *in vivo* that $PGE_2$ specifically repressed colonic CD103$^-$CD11b$^+$ MNPs dependently of gut microbiota, while CD103$^+$ MNPs were unaffected. A recent paper by Nakahashi-Oda. C, *et al* (2016) has suggested that CD103$^-$ MNPs are also able to boost proliferation of Foxp3$^+$ Tregs within the intestine, ability of which is suppressed by markers on dying epithelial cells [6].

Therefore, to determine whether these MNPs subtypes had a similar effect on iTreg induction *in vitro*, on day 0, naïve T cells (CD4$^+$CD25$^-$Foxp3-YFP$^-$) were sorted from Foxp3-YFP mice splenocytes, and MNPs (CD45$^+$CD3$^-$CD11c$^+$MHCII$^+$CD11b$^+$ and CD103$^+$ or CD103$^-$) from either WT or Foxp3-YFP mice mLNs. CD103$^-$ and CD103$^+$ MNPs were co-cultured in a 1:2 ratio with naïve T cells plus TGFβ, IL-2 and soluble aCD3 until day 5, then cells were stained for Treg markers (**Fig. 5.5a**). **Figure 5.5b** demonstrates the gating strategy for CD25, Foxp3 and RORγt in live CD3$^+$CD11c$^-$ cells.

As expected, there was a greater proportion of Foxp3$^+$ Tregs in the co-culture with CD103$^+$ MNPs, however surprisingly within this population there was lower RORγt expression compared to what was observed with the naïve T cells co-cultured with CD103$^-$ MNPs (**Fig. 5.5c**). This suggests that the increased proportion of CD103$^-$ CD11b$^+$ MNPs within the colon of indomethacin-treated mice are important for enhancing the population of RORγt$^+$ Tregs.

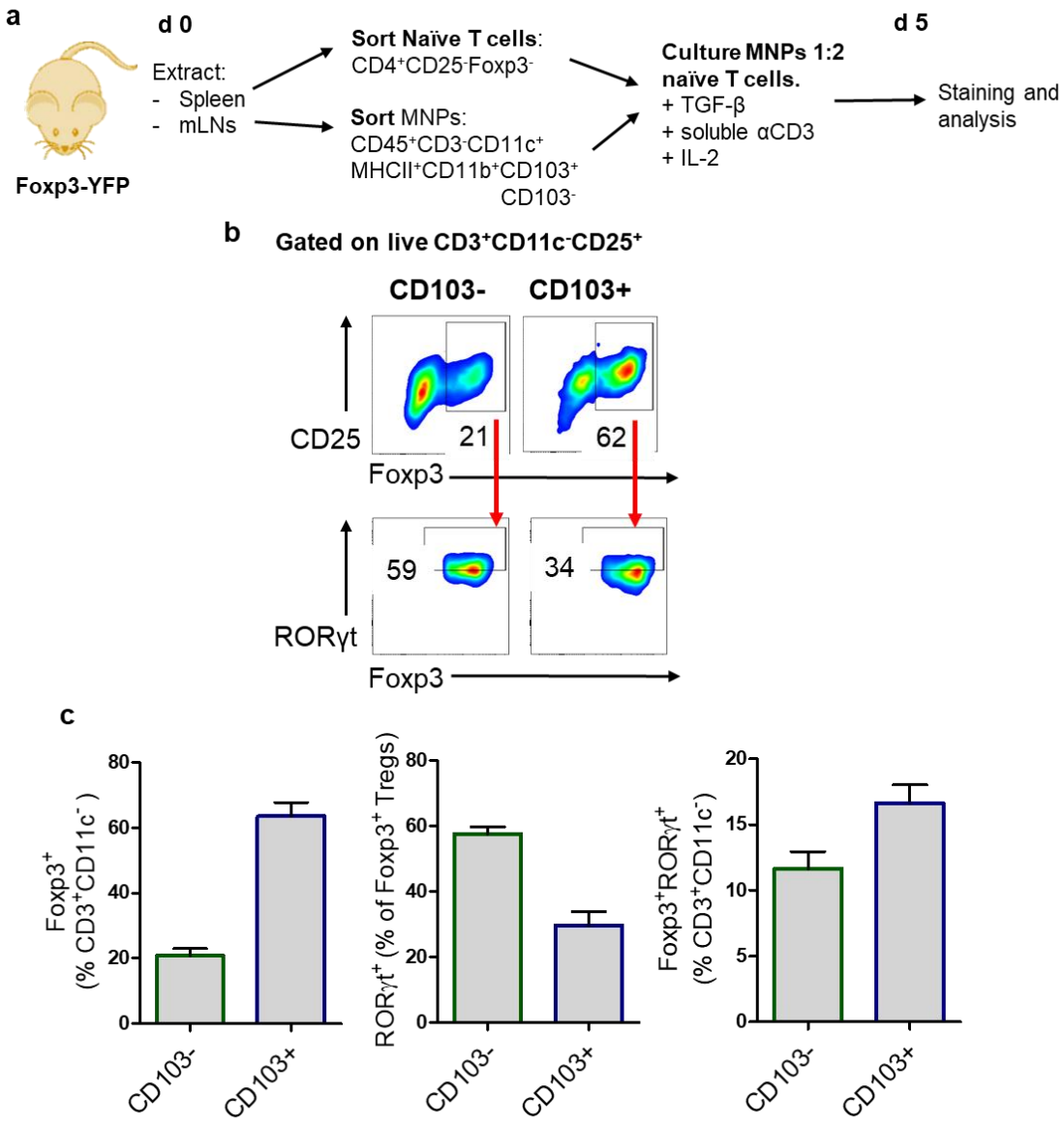


**Figure 5.5: Effect of MNPs on Treg induction *in vitro*.** *(a) Experimental timeline for sorting naïve T cells from the spleens of Foxp3-YFP mice, and CD103$^+$ or CD103$^-$ MNPs from mLNs. MNPs cultured in a 2:1 ratio to naïve T cells, with TGF-β, soluble αCD3, IL-2 on d 0. Cells were stained with anti-Foxp3 and anti-RORγt antibody and analysed on d 5. (b) Flow cytometry plot illustrating the gating strategy for Foxp3 and RORγt in live CD3$^+$CD11c$^-$CD25$^+$ cells cultured with either CD103$^+$ or CD103$^-$ MNPs. (c) Graphs show proportion of Foxp3$^+$ and RORγt$^+$Foxp3$^+$ cells within live CD3$^+$CD11c$^-$CD25$^+$ cells. (Mean ± SEM of triplicates.) Data represents one experiment. Representative of three independent experiments.*

### 5.3.6 Caecum content from indomethacin-treated mice enhance CD103$^-$ MNPs induction of RORγt$^+$Foxp3$^+$ Tregs

It was observed *in vivo* that $PGE_2$ specifically repressed colonic CD103$^-$ MNPs dependently of gut microbiota, while CD103$^+$ MNPs were unaffected (**Fig 5.3c, d**). Therefore, to determine whether the metabolites produced by gut microbiota affected the CD103$^-$CD11b$^+$ MNPs ability to induce RORγt$^+$Foxp3$^+$ iTregs, naïve T cells (CD4$^+$CD25$^-$Foxp3-YFP$^-$) were sorted from Foxp3-YFP mouse splenocytes, and CD103$^-$ MNPs from mLNs. MNPs and naïve T cells were co-cultured for five days with TGFβ, soluble αCD3 and IL-2. On day 1, sterilely filtered caecum matter from vehicle- or indomethacin-treated mice was added. Day 5, cells were stained for Treg markers (**Fig. 5.6a**). Flow cytometry dot plot Foxp3 and RORγt expression is demonstrated in **figure 5.6b**. The indomethacin-treated mice sterilely filtered caecum content did not affect Foxp3 expression within the CD103$^-$ MNPs co-culture, however there was an increase in the RORγt expression within this Foxp3$^+$ population (**Fig. 5.6c**). These data suggest that indomethacin-treated mice sterilely filtered caecum can further enhance RORγt$^+$Foxp3$^+$ Treg induction by CD103$^-$ MNPs *in vitro.*

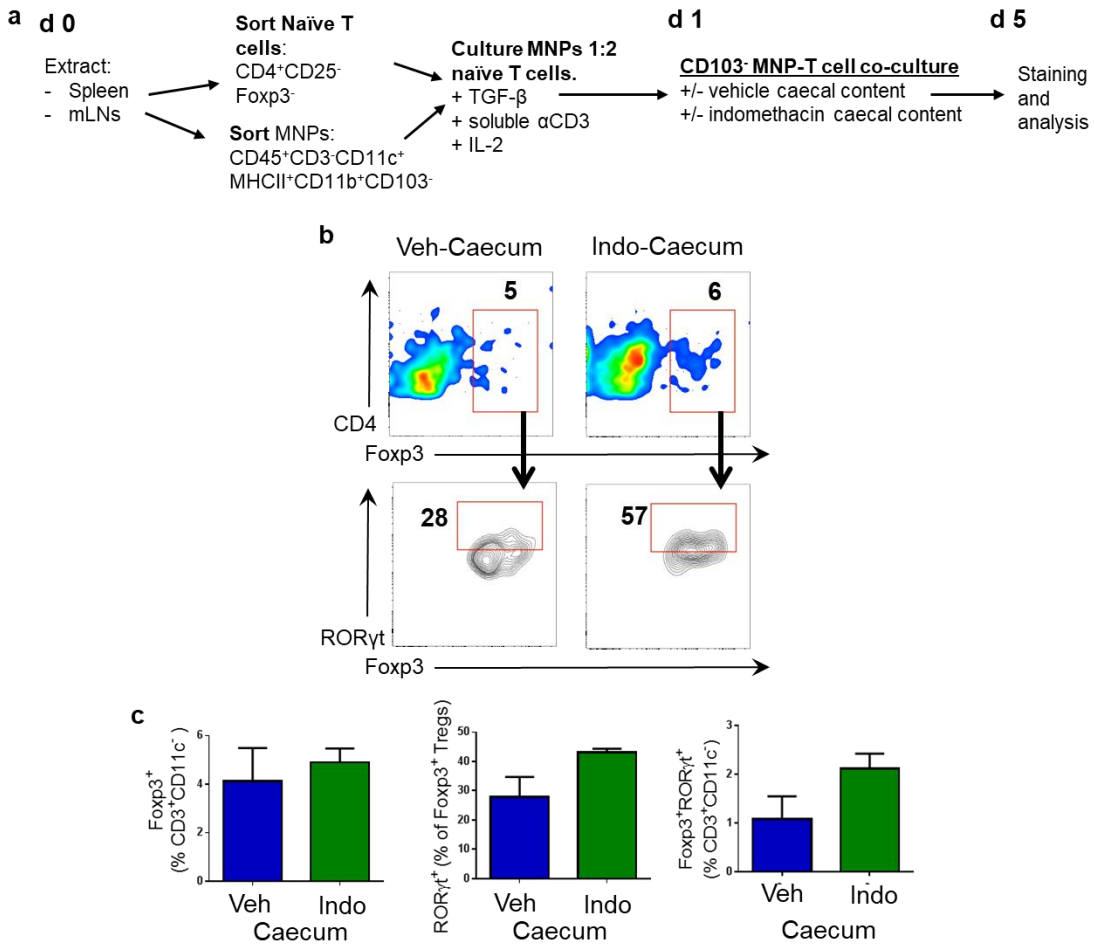


**Figure 5.6: Effects of caecum content on a sorted CD103$^-$ MNP-naïve T cell co-culture.** *(a) Experimental timeline for sorting of naïve T cells from the spleens of Foxp3-YFP mice, and CD103$^-$ MNPs from mLNs. MNPs cultured in a 2:1 ratio to naïve T cells, with TGF-β, soluble αCD3, IL-2 on d 0, and sterilely filtered caecum matter from vehicle or indomethacin-treated mice was added on d 1. Cells were stained with anti-Foxp3 and anti-RORγt antibody and analysed on d 5. (b) Flow cytometry plot illustrating the gating strategy for Foxp3 and RORγt in live CD3$^+$CD11c$^-$CD25$^+$ cells cultured with CD103$^-$ MNPs with sterilely filtered caecum content from mice treated with either vehicle or indomethacin. (c) Graphs show proportion of Foxp3$^+$ and RORγt$^+$Foxp3$^+$ cells within live CD3$^+$CD11c$^-$CD25$^+$cells. (Mean ± SEM of triplicates.) Data represents one experiment. Representative of three independent experiments.*

### 5.3.7 Colonic CD103$^-$ MNPs from indomethacin-treated mice express higher levels of IFN-β related genes than vehicle-treated mice

Multiple groups have suggested that CD103$^-$ DCs can induce Tregs via retinoic acid and TGFβ production, similar to the mechanism CD103$^+$ DCs use [6, 180-182]. As previously shown in **figure 5.3c**, **d** indomethacin-treated mice had a greater proportion of CD103$^-$ cLP MNPs within the colonic tissue compared to the vehicle group, and co-culturing CD103$^-$ cLP MNPs with naïve T cells resulted in a greater proportion of RORγt$^+$Foxp3$^+$ Tregs compared to the cells cultured with CD103$^+$ cLP MNPs (**Fig. 5.5c**).

To examine whether the cLP MNP subset which was enhanced in the colons of indomethacin-treated mice were also expressing IFN-β, which is known to enhance proliferation of Tregs, WT C57BL/6 mice were treated with vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) for 4 days, colons extracted and cells isolated. cLP cells were sorted for CD45$^+$CD3$^-$ CD11$^+$MHCII$^+$CD103$^-$ mNPs, then processed for PCR (**Fig. 5.7a**). The mNP sorting gating strategy is demonstrated in **Fig. 5.7b**.

The PCR results showed that CD103$^-$ MNPs extracted from colons of indomethacin-treated mice have both a two-fold increase of *Ifn-b* and *Ifn-a* compared to the vehicle-treated group, and also a larger increase in *Irf7* (5.5X) (**Fig. 5.7c**). IFN-β can act in both an autocrine and paracrine manner, resulting in a positive feedback loop. *Irf7* is downstream of the interferon receptor and forms a complex with p-STAT-1 and p-STAT-2 after receptor activation. IRF7 is largely restricted to lymphoid tissue and activates viral-inducible cellular genes such as *Ifn-b,* hence an increase in one gene suggests that there will be an increase in the other.

The p-STAT-1/p-STAT-2 and IRF7 complex translocates to the nucleus, and upregulates the interferon-stimulated gene *(Isg)15*, levels of which were 4x greater in the CD103$^-$ cLP MNPs from the indomethacin-treated mice colons, compared to the vehicle group (**Fig. 5.7c**). ISG15 conjugates to intracellular target proteins after activation by signal proteins such as IFN-β or IFN-α transmitting the downstream effect (**Fig. 5.7c**) [74]. These data demonstrates that inhibition of $PGE_2$ enhances colonic CD103$^-$CD11b$^+$ cLP MNPs type 1 interferon gene expression.

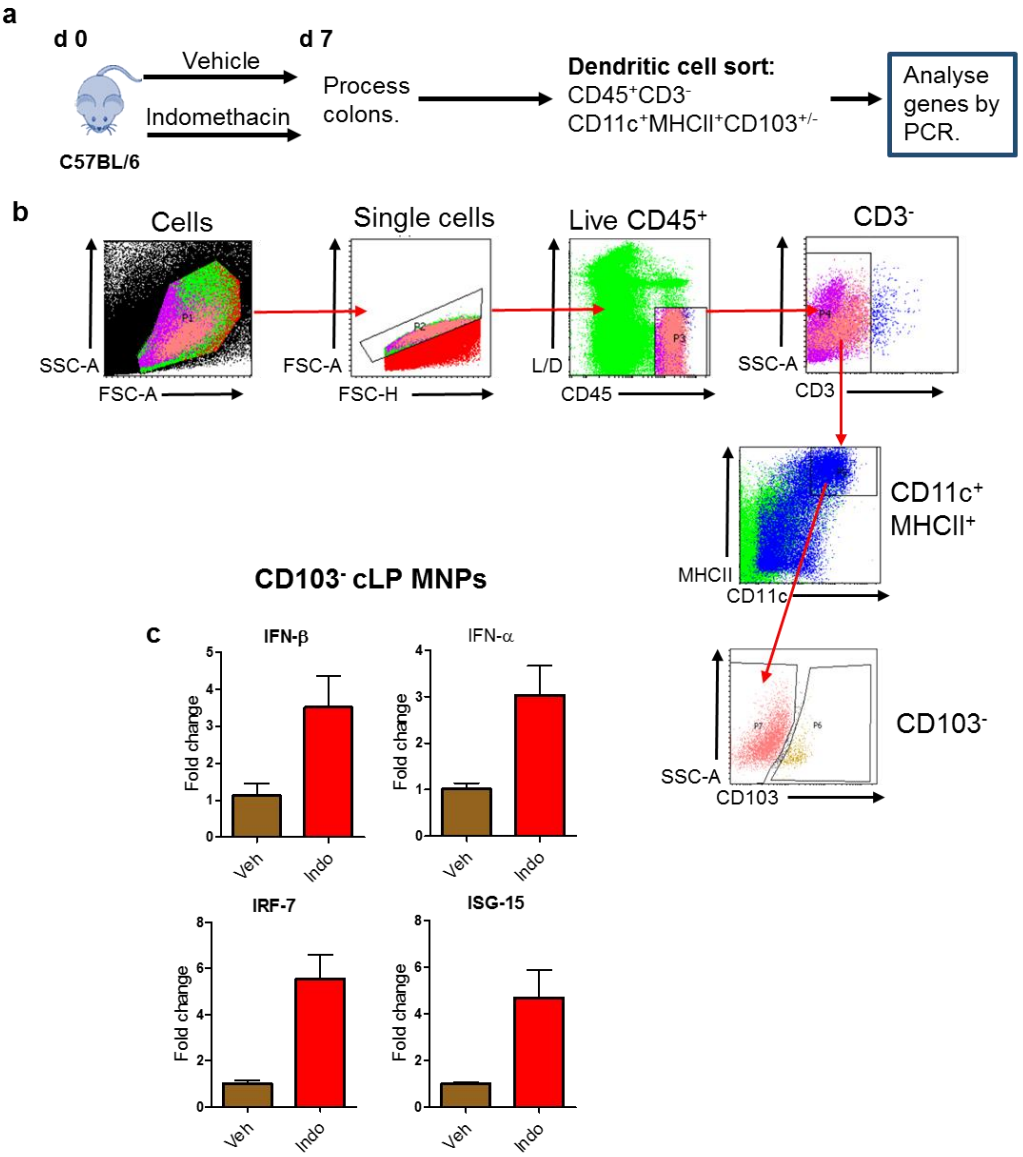


**Figure 5.7: Type 1 interferon gene expression in colonic CD103$^-$ MNPs.** *(a) Experimental timeline for sorting CD103$^-$ MNPs from colons of C57BL/6 mice treated with either vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day). (b) Flow cytometry plot illustrating the gating strategy for sorting CD103$^-$ MNPs from colon tissues. (c) Graphs to show the fold change of various IFN-β-related genes compared to the housekeeping gene, GAPDH in colonic CD103$^-$ MNPs. Data shown as means ± SEM (error bars), samples pooled from 3-4 mice. Data representative of three independent experiments.*

### 5.3.8 Sterile filtered caecum content from indomethacin-treated mice induces type 1 interferon gene expression in BMDCs

Microbial products from indomethacin treated mice had a greater ability to enhance RORγt$^{+}$ expression in Tregs compared to vehicle treated microbial products, thus it was thought that culturing BMDCs with caecum from indomethacin-treated mice would result in tolerogenic BMDCs. Therefore, BM cells were extracted from WT mice and cultured for three days with GM-CSF, media refreshed every three days until day 9. Cells were sorted for CD45$^{+}$CD3$^{-}$MHCII$^{+}$CD103$^{-}$ BMDCs, then cultured for 2.5 or 6 hours with either media, or sterilely filtered caecum content from vehicle, indomethacin, or indomethacin plus an EP4 agonist, treated mice. Cells were washed three times in PBS/2 % FCs, then re-suspended in β-ME and buffer RLT and stored at -80 $^{o}$C for future use (**Fig. 5.8a**). Cells were lysed and cDNA produced prior to PCR to detect various genes; *Ifn-β, Irf-7* and *Isg-15*. Culturing BMDCs with sterilely filtered caecum content resulted in a greater proportion of gene expression compared to the BMDCs cultured with media only (**Fig. 5.8b, c**). After both 2.5 and 6 hours culturing BMDCs with sterilely filtered caecum content from vehicle or indomethacin treated mice, there was an increase in type 1 interferon genes, whereas this was not observed with the cells cultured with the filtrates from indomethacin plus an EP4 agonist treated mice (**Fig. 5.8b, c**).

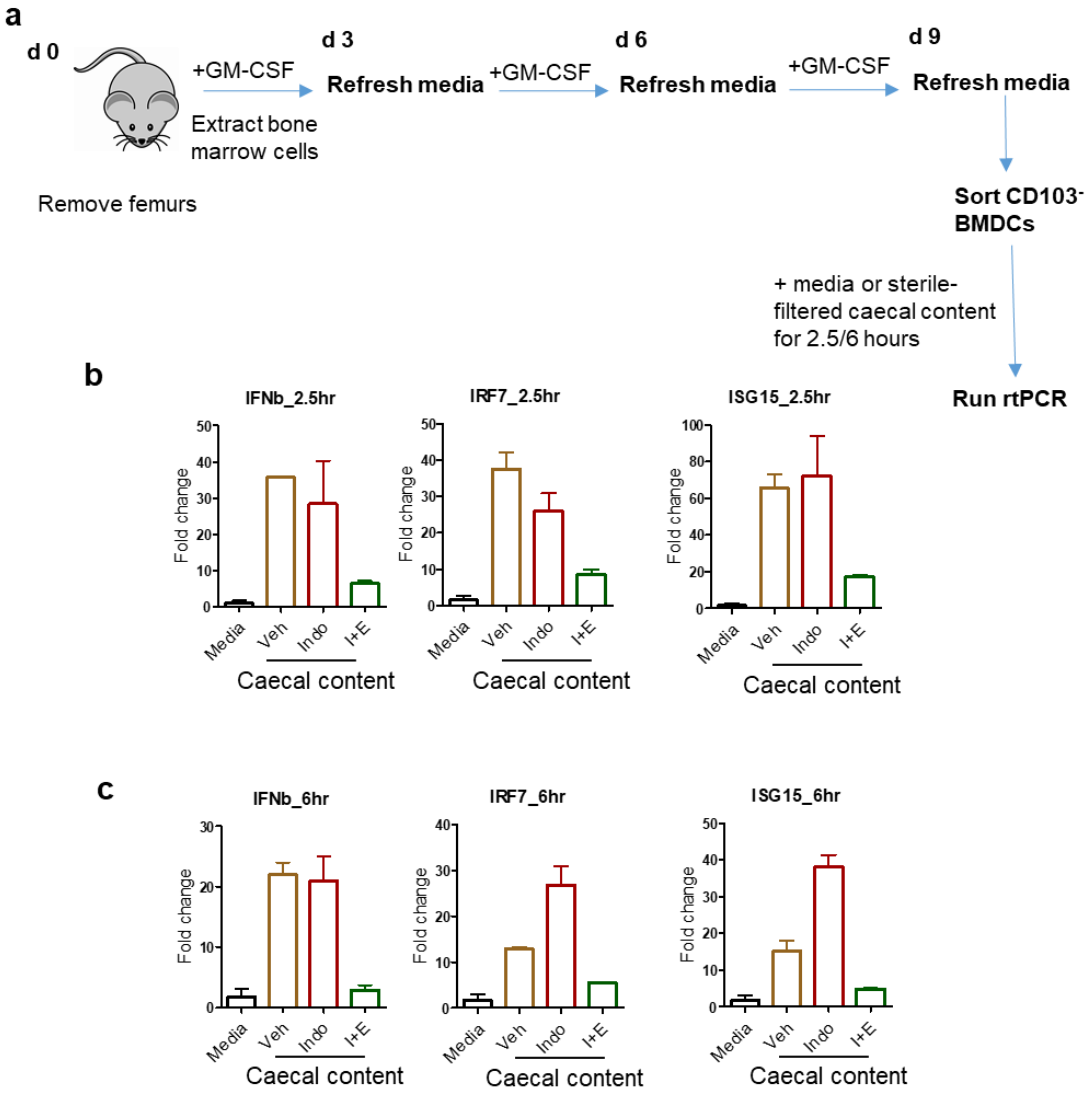


**Figure 5.8: The effect of sterile filtered caecum content on BMDC differentiation**. *(a) Experimental timeline for BMDC differentiation. Femurs were removed from mice on d 0, and bone marrow cells were cultured with media plus GM-CSF for nine days. BMDCs were sorted for CD103$^-$ DCs and cultured with either media, or sterilely filtered caecum content from mice treated with vehicle, indomethacin or indomethacin plus an EP4 agonist, for 2.5 or 6 hours, cells washed then sorted for PCR. (b) Type 1 interferon gene fold change compared to the housekeeping gene GAPDH after culturing CD103$^-$ DCs for 2.5 hours with media, or sterilely filtered caecum content from vehicle, indomethacin or indomethacin plus an EP4 agonist treated mice. (c) Type 1 interferon gene fold change compared to the housekeeping gene GAPDH after culturing CD103$^-$ DCs for 6 hours with media, or sterilely filtered caecum content from vehicle, indomethacin or indomethacin plus an EP4 agonist treated mice. Data shown as means ± SEM (error bars), n = 4-8, samples from two independent experiments.*

### 5.3.9 Inhibition of endogenous $PGE_2$ increases STAT-1 phosphorylation in both colonic Tregs and MNPs

Colonic $CD103^-$ MNPs from indomethacin-treated mice had an increase in both IFN-β and type 1 interferon genes detected by PCR (**Fig. 5.7c**). To confirm that there was also increased interferon signalling, not just an increase in gene expression, phosphorylation of STAT-1, downstream of the interferon receptor was measured via flow cytometry in both Tregs and cLP MNPs.

Mice were treated as before for seven days with either vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) then colons processed and stained for both Treg and MNPs markers and p-STAT-1 and 2, then ran on the flow cytometer (**Fig. 5.9a**). The flow cytometry dot plots for CD25, Foxp3 expression and p-STAT-1 is demonstrated in **figure 5.9b**. As observed previously, there was a greater number of $Foxp3^+$ Tregs within the colon of indomethacin treated mice, compared to mice treated with vehicle only ($p \leq 0.01$) (**Fig. 5.9b**). Within this $Foxp3^+$ population, there was likewise a significantly higher number of p-STAT-1 positive cells ($p \leq 0.01$) (**Fig. 5.9c**). This suggested that there was increased type 1 interferon signalling within the colon Tregs of the indomethacin treated mice, either stabilising gene expression, or boosting proliferation [6].

STAT phosphorylation was also detected within the cLP MNP population. The gating strategy for $CD11b^+$ and consequent p-STAT-1 expression within live $CD45^+CD3^-$ $CD11c^+$ MNPs is demonstrated in **figure 5.9d**. There was a greater number of $CD11b^+$ MNPs within the colons of indomethacin treated mice (**Fig. 5.9d**), and within this population there was a significantly greater number of p-STAT-1 positive cells ($p \leq 0.05$) (**Fig. 5.9e**). This supports the previous data that there was increased IFN-β and type 1 interferon-related genes within the colons of indomethacin treated mice, as there was also increased down-stream signalling from the receptor, measured via STAT-1 phosphorylation. Type 1 IFN related proteins signal in a positive feedback loop, indicating that a trigger to begin IFN-β production can then maintain its expression via its feedback loop, acting both in a paracrine and autocrine fashion.

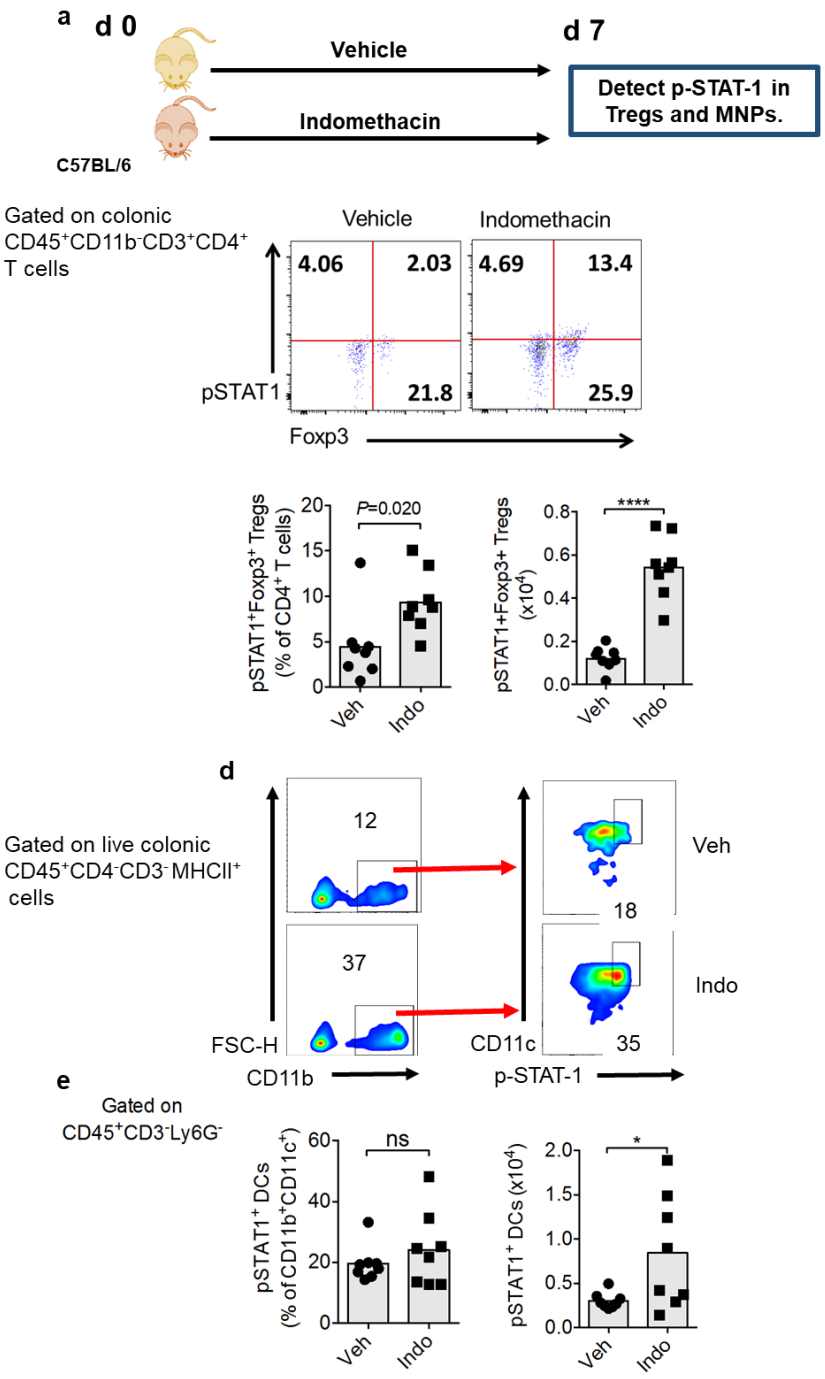


**Figure 5.9: Detection of type 1 interferon signalling in colonic MNPs and Tregs.** *(a) Experimental timeline for in vivo inhibition of endogenous $PGE_2$. WT C57Bl/6 mice were treated with either vehicle, or indomethacin in drinking water for 7 days. Mice were culled on d 7, colons removed and processed. Cells were stained with antibodies for phosphorylated STAT-1 within MNPs or Treg cell populations. (b) Gating strategy for Foxp3$^+$ Tregs and phosphorylation of STAT-1 within this population. (c) Proportion and number of p-STAT-1 positive Foxp3$^+$ colonic Tregs from indomethacin or vehicle treated mice. (d) Gating strategy for CD11b$^+$ MNPs and phosphorylation of STAT-1 within this population. (e) Proportion and*

*number of p-STAT-1 positive CD11b$^+$ MNPs from indomethacin or vehicle treated mice. Data shown as means ± SEM (error bars) are pooled from two independent experiments. (n = 6 - 8) *P ≤ 0.05, **P ≤ 0.01 ***P ≤ 0.001 by Mann-Whitney U.*

### 5.3.10 IFNAR knockout prevents an increase in colonic RORɣt$^+$ Tregs after inhibition of endogenous $PGE_2$

Previous data showed that there were increased type 1 interferon related genes, and subsequent STAT signalling in colonic tissue of indomethacin treated mice, compared to mice treated with vehicle only. Therefore, to confirm that $PGE_2$ was influencing interferon signalling to affect colonic Treg induction, WT C57BL/6 or IFNAR knock-out mice were treated with vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) in drinking water for five days, then culled and colons processed for flow cytometry (**Fig. 5.10a**). The flow cytometry dot plot for CD25, Foxp3 and RORγt expression in live CD45$^+$CD3$^+$CD4$^+$ cells is demonstrated in **figure 5.10b**. As previously observed, in WT C57BL/6 mice, inhibiting $PGE_2$ production via indomethacin resulted in a significantly greater proportion and number of Foxp3 expressing cells ($p \leq 0.01$) There was no difference observed between the colons of the IFNAR knock-out indomethacin or vehicle treated mice (**Fig. 5.10c**). This was similarly observed with RORγt expression. Within the Foxp3$^+$ population, there was a significantly greater proportion RORγt expressing cells in the indomethacin treated mice colons, compared to the vehicle treated group ($p \leq 0.01$) There was no difference observed between the indomethacin and vehicle treated IFNAR mice colons (**Fig. 5.10d**).

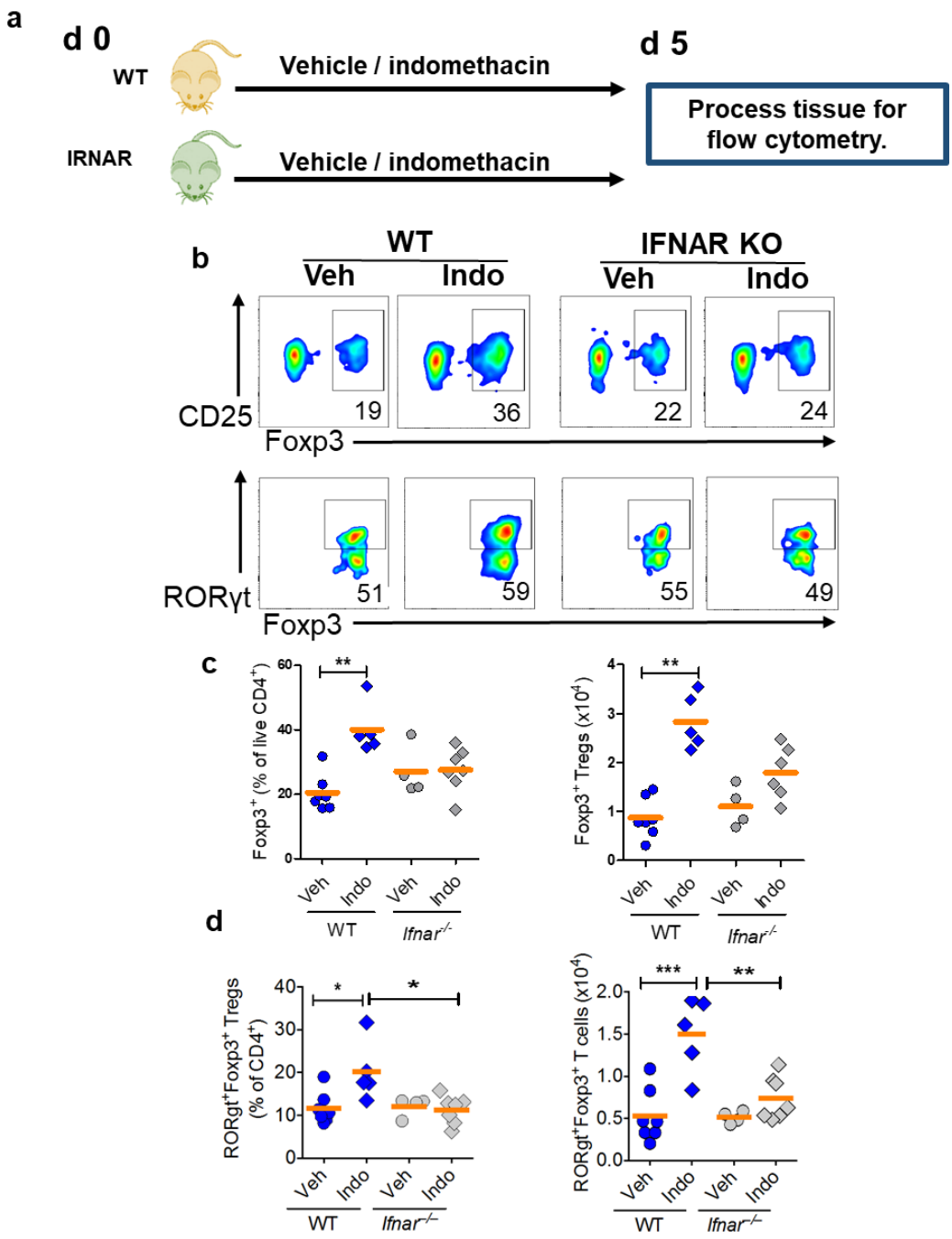


**Figure 5.10: Confirmation of the IFN pathway in Treg induction**. *(a) Experimental timeline for in vivo inhibition of endogenous $PGE_2$. IFNAR knock-out or WT C57Bl/6 mice were treated with either vehicle, or indomethacin in drinking water for 4 days. Mice were culled on d 4, colons removed and processed. Cells were stained with Treg antibodies. (b) Flow cytometry gating strategy for $Foxp3^+$ and $ROR\gamma t^+$ Tregs within live colonic $CD45^+CD3^+CD4^+$ T cells. (c) Graphs show percentage and number of $Foxp3^+$ Tregs within colons of treated mice. (d) Graphs show percentage and number of $ROR\gamma t^+$ Tregs within colons of treated mice. Data*

*shown as means ± SEM (error bars) are pooled from two independent experiments. (n = 4 - 7) *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 by Kruskal-Wallis test, Dunn's test used for post-hoc test.*

### 5.3.11 Inhibition of endogenous $PGE_2$ is unable to influence $CD103^-$ cLP MNPs due to IFNAR KO

It was shown that there was an increase in type 1 interferon genes in the $CD103^-CD11b^+$ MNPs within the colons of indomethacin treated mice compared to the vehicle treated mice, and this was the only subset that was affected by inhibition of $PGE_2$. It was also observed that $ROR\gamma t^+Foxp3^+$ Tregs were unaffected by $PGE_2$ inhibition when the interferon signalling pathway was inhibited. Therefore, to see whether the $CD103^-CD11b^+$ cLP MNP population was similarly affected by inhibition of interferon signalling, as before WT C57BL/6 or IFNAR knockout mice were treated with vehicle (0.5 % ethanol) or indomethacin (5 mg/kg/day) in the drinking water for five days. Mice were culled and colons processed for flow cytometry (**Fig. 5.11a**). Flow cytometry dot plot for CD103 and CD11b expression in live $CD45^+CD3^-CD11c^+MHCII^+$ cells is demonstrated in **figure 5.11b**. As observed before, there was an increase in $CD103^-CD11b^+$ MNPs in the colons of WT mice treated with indomethacin compared to WT mice treated with vehicle ($p \leq 0.001$). There was no difference observed between the $CD103^-CD11b^+$ MNP subpopulation of the IFNAR knockout mice treated with either vehicle or indomethacin (p = NS) (**Fig. 5.11c**).

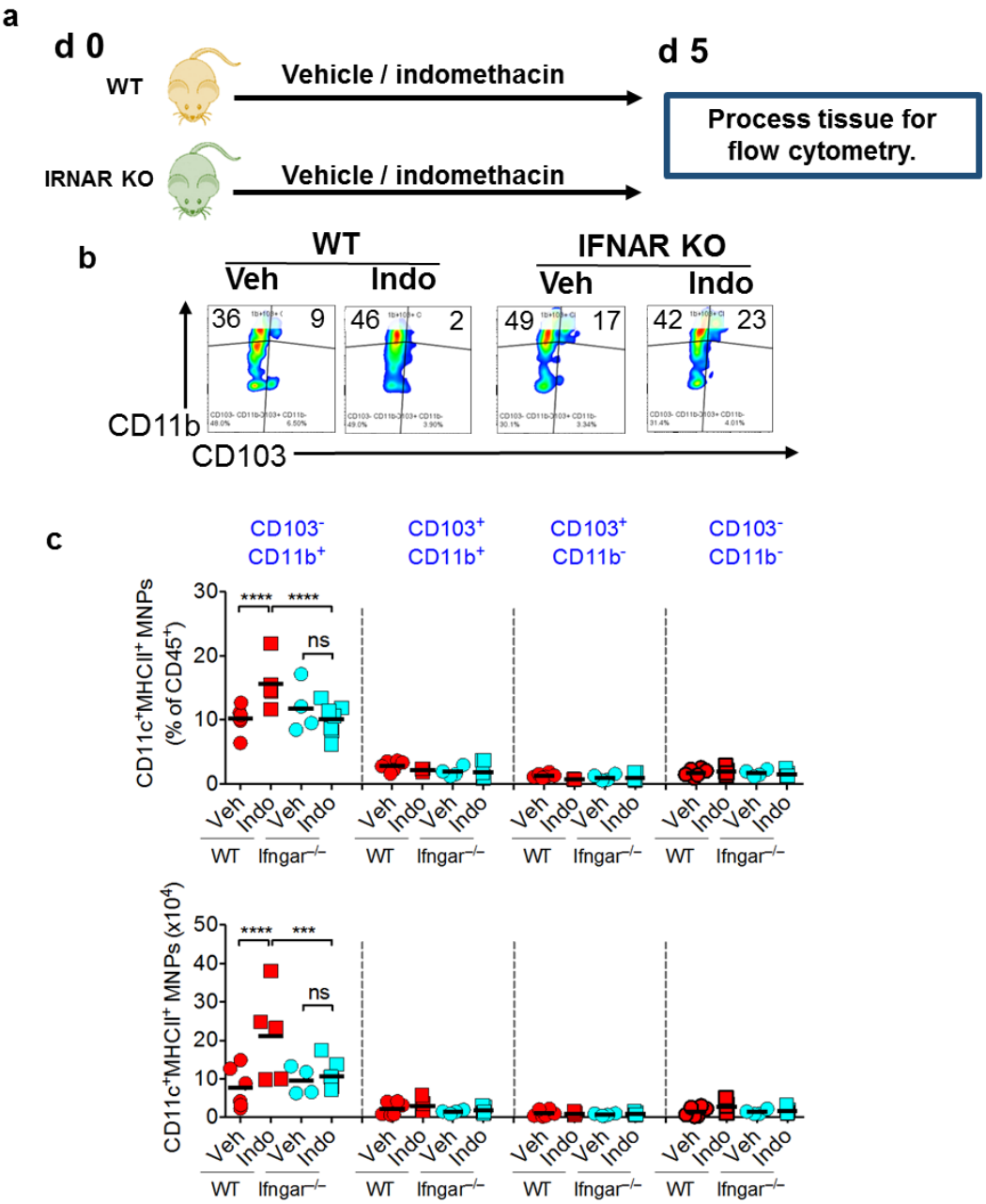


**Figure 5.11: Confirmation of the IFN pathway in cLP MNP subsets**. (*a) Experimental timeline for in vivo inhibition of endogenous $PGE_2$. IFNAR knock-out or WT C57Bl/6 mice were treated with either vehicle, or indomethacin in drinking water for 4 days. Mice were culled on d 4, colons removed and processed. Cells were stained with Treg antibodies. (b) Flow cytometry gating strategy for $CD103^{+/-}$ cLP MNPs within live colonic $CD45^{+}CD3^{-}B220^{-}CD11c^{+}MHCII^{+}$ cells. (c) Graphs show number of $CD103^{+}$ or $CD103^{--}$ MNPs within colons of IFNAR KO and WT mice. Data shown as means ± SEM (error bars) are pooled from two independent experiments. (n=5-7) *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, ****p ≤ 0.0001 by Kruskal-Wallis test, Dunn's test used for post-hoc test.*

### 5.3.12 Indomethacin does not affect iTreg differentiation

A question was raised about whether indomethacin remained within the caecum of mice after treatment, and this influenced Treg induction, rather than due to the resulting products from inhibition of prostaglandins. Therefore, naïve T cells were sorted from the spleens of a Foxp3-YFP$^+$ mouse, and cultured for 24 hours before addition of media, vehicle, $PGE_2$ or indomethacin. On day 4 cells were stained and analysed for Foxp3 expression and samples ran on the flow cytometer (**Fig. 5.12a**). Gating strategy for Foxp3 expression is demonstrated in **figure 5.12b**. Graphs show the proportion and number of Foxp3$^+$ expressing cells cultured with media vehicle, $PGE_2$ or indomethacin (**Fig. 5.12c**). Addition of indomethacin does not influence Foxp3 expression within the cell culture, there was no difference observed between the cells cultured with media or vehicle, however as expected, $PGE_2$ inhibited Foxp3 expression (**Fig. 5.12c**). This confirms that indomethacin is not directly affecting naïve T cell Foxp3 expression *in vivo*.

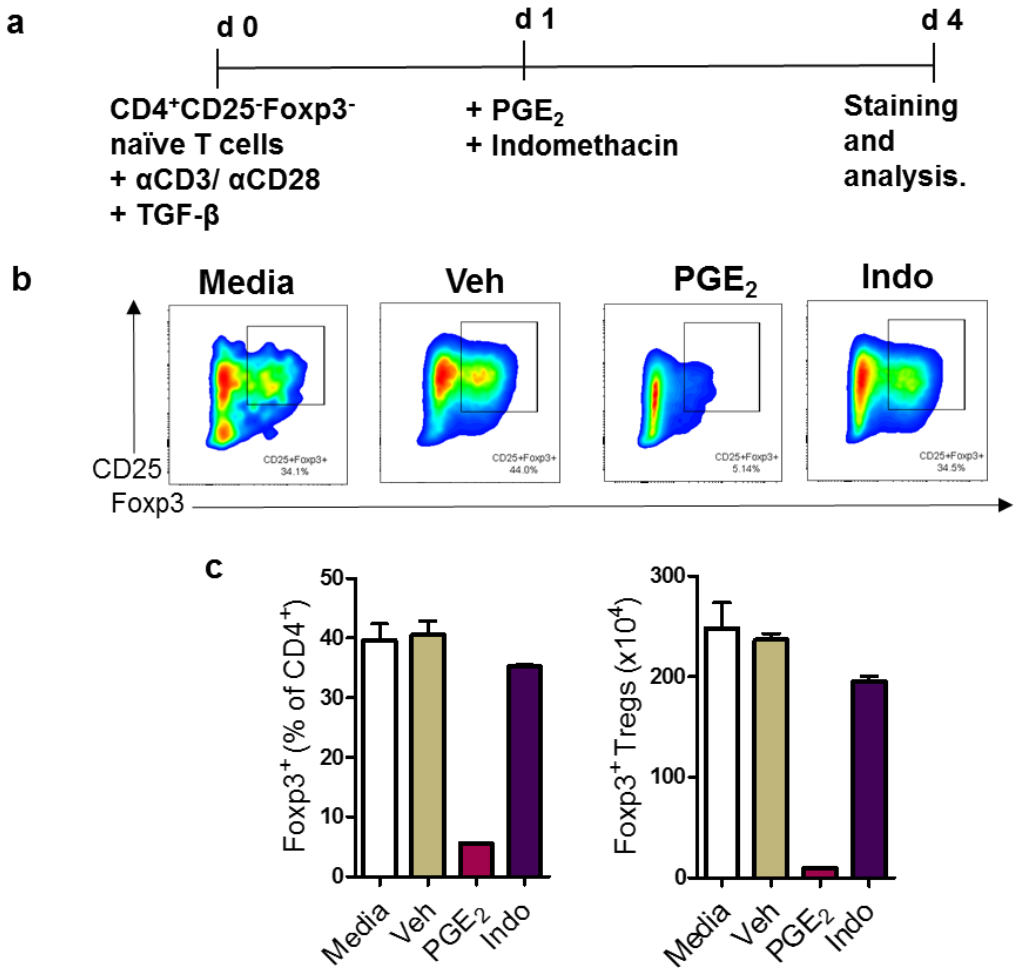


**Figure 5.12: The effect of indomethacin on naïve T cell differentiation.** (*a) Experimental timeline of sorted naïve T cells cultured with $PGE_2$ and indomethacin added on d 1. Cells stained for anti-mouse Foxp3, and samples run on the Fortessa on d 4. (b) Flow cytometry plot illustrating the gating strategy for CD25, Foxp3 and Ki67 in live $CD4^+$ T cells. (c) Proportion and number of $Foxp3^+$ Tregs from naïve T cells cultured with media, vehicle, $PGE_2$ or indomethacin. Data shown as means ± SEM (error bars). Data represents one experiment. Representative of three independent experiments.*

## 5.4 Discussion

At this point, I have shown that colonic RORγt$^+$ Tregs were significantly increased after inhibition of endogenous $PGE_{2,}$ and this involved the gut microbiota. Additionally, colonic CD11b$^+$CD103$^-$ MNPs are enhanced through inhibition of endogenous $PGE_2$ and this is also influenced by gut microbiota, as it can be inhibited by antibiotic treatment and was not observed in MyD88/TRIF DKO mice. The colonic CD11b$^+$CD103$^-$ MNPs from indomethacin treated mice express a greater proportion of type 1 interferon genes, which correlates with increased phosphorylation of STAT-1 in both Tregs and MNPs. Interferon signalling is important for the induction of both colonic Tregs and CD11b$^+$CD103$^-$ MNPs, as there was no increase in these cell types in indomethacin treated IFNAR KO mice.

Numbers of colonic RORγt$^+$ Tregs were increased in T cell specific EP4 conditional KO mice, whereas Foxp3 levels were unaffected by the disruption of $PGE_2$ signalling in T cells, contradictory to what was observed in the *in vitro* results (**chapter 3, figure 3.4**). These findings suggested that there are other factors involved in $PGE_2$'s suppression of colonic Tregs *in vivo*. Previous work has demonstrated that an increased commensal microbiota correlates with augmented colonic Tregs [51, 55, 171]. Since the microbiome has been shown to be so important to the induction and regulation of the immune system, it was then thought that possibly the microbiota was involved in $PGE_2$'s suppression of colonic Tregs. Suppression of endogenous $PGE_2$ did not affect colonic Tregs in mice with depleted gut microbiota, suggesting that the microbiota mediates $PGE_2$ suppression of RORγt$^+$ Tregs (**Fig. 5.1**). It has been suggested that antibiotic treatment can affect the ability to metabolise indomethacin, largely due to reduction in β-glucuronidase activity which results in increased levels of prostanoids in the blood, compared to indomethacin-only treated mice [183]. However, as previously mentioned, the antibiotic-only treated group had similar levels of Tregs compared to the vehicle-only treated group, so if there had been an increase in $PGE_2$, this did not result in any further suppression of colonic Tregs (**Fig. 5.1**). It is trusted that the dose and length of time that the antibiotics were used are enough to significantly deplete gut microbiota. The antibiotic combination and length of treatment has been used by multiple groups. Fukisaka. S. *et al* (2016), saw a significant reduction of bacterial DNA in the faeces, suggesting that the fortnight of treatment will be enough to significantly reduce the intestinal bacterial burden [54, 112].

In addition to this, inhibition of COX pushes arachidonic acid down the leukotriene pathway via lipoxygenase [184]. Leukotrienes attract neutrophils, which also express β-glucuronidase, to aid in indomethacin metabolism. There was an increased number of neutrophils detected in the colons of the indomethacin treated mice, suggesting that the compound is still effectively metabolised and suppressing COX activity (**Fig. 5.1**) [184].

To further confirm the hypothesis that $PGE_2$ modulates the commensal microbiota to influence colonic RORɣt$^+$Foxp3$^+$ Treg development, MyD88/TRIF DKO mice were used. MyD88 and TRIF are signal adapter molecules for TLRs, which are important components of APCs to recognise bacterial signature markers and induce a relevant immune response [185]. Indomethacin-treated WT mice had a greater number of colonic RORɣt$^+$ Tregs compared to vehicle-treated WT mice, however this difference was not observed between MyD88/TRIF DKO mice (**Fig. 5.2**). This supports the idea that colonic RORɣt$^+$Foxp3$^+$ Tregs are affected by $PGE_2$'s modulation of gut microbiota and subsequent metabolites. These data confirm that $PGE_2$ regulates colonic RORγt$^+$ Tregs via the gut microbiota.

There are two main subsets of colonic MNPs, CD103$^+$ MNPs which are considered to typically enhance Foxp3$^+$ Treg differentiation through production of retinoic acid and TGFβ, and CD103$^-$ MNPs which have been thought to push a more pro-inflammatory phenotype [68, 173]. However recently, Nakahashi-Oda, C. *et al* (2016), demonstrated that gut microbiota stimulated colonic CD11b$^+$CD103$^-$ DCs production of IFN-β which enhanced proliferation of Tregs [6]. MNPs are vital for detecting microbial metabolites and inducing immune cells, therefore it was examined whether subsets were also affected by inhibition of endogenous $PGE_2$. $PGE_2$ inhibition enhanced CD11b$^+$CD103$^-$ cLP MNPs compared to vehicle treated mice and this was dependent on microbiota. Co-treatment of antibiotics and indomethacin prevented an increase in this cLP MNP subset (**Fig 5.3**). The involvement of microbiota is supported by Nakahashi-Oda, C. *et al* (2016) who saw that faecal matter stimulated BMDC IFNβ production in *in vitro* cell cultures [6].

To further confirm that $PGE_2$'s inhibition of $CD11b^+CD103^-$ cLP MNPs was influenced by the microbiota, MyD88/TRIF DKO mice were used. As previously observed in **figure 5.3**, inhibition of endogenous $PGE_2$ in WT mice resulted in a significantly greater number of $CD11b^+CD103^-$ cLP MNPs compared to indomethacin-treated MyD88/TRIF DKO mice, supporting the idea that $PGE_2$ influences microbial metabolite production consequently affecting $CD11b^+CD103^-$ cLP MNPs development (**Fig. 5.4**). There was no difference observed between the $CD103^+$ or $CD103^-CD11b^-$ cLP MNP groups.

Inhibition of endogenous $PGE_2$ had the greatest effect on colonic $CD103^-$ MNP levels, so wanted to determine whether this affected naïve T cell differentiation differently to $CD103^+$ MNPs. Initial results confirmed previous literature, $CD103^+$ DCs induced the greatest proportion of Foxp3 in naïve T cells, however within this $Foxp3^+$ population, the cells cultured with $CD103^-$ DCs had higher $ROR\gamma t^+$ expression (**Fig. 5.5**). $CD103^-$ DCs Treg induction is dependent on microbiota, so it is logical that $PGE_2$'s influence on the microbiota and metabolite composition will have a greater effect on this subset [186].

In **figure 5.5,** it was shown that although naïve T cells cultured with $CD103^-$ MNPs expressed less Foxp3 than cells cultured with $CD103^+$ MNPs, they expressed more RORγt. *In vivo* this involved the gut microbiota therefore it was questioned whether addition of sterilely filtered caecum content from indomethacin treated mice could further enhance $CD103^-$ MNPs ability to induce $ROR\gamma t^+$ Tregs. The $CD103^-$ MNP co-culture with sterilely filtered caecum content from indomethacin-treated mice had a greater proportion of $ROR\gamma t^+Foxp3^+$ Tregs, compared to the co-culture with sterilely filtered vehicle caecum content, suggesting that the metabolites present in the caecum content of mice treated with indomethacin enhanced the regulatory ability of the $CD103^-$ MNPs (**Fig. 5.6**).

Nakahashi-Oda, C. *et al* (2016) demonstrated that colonic $CD103^-$ DCs produced IFN-β and this was shown to increase Treg proliferation, therefore IFN-β and IFN-β-related genes were measured in $CD103^-$ MNPs sorted from colons of vehicle- or indomethacin-treated mice. There was a greater fold change of type 1 interferon genes such as; *Ifn-β, Ifn-α, Irf-7 and Isg-15*, in the indomethacin-treated mice compared to vehicle-treated mice (**Fig. 5.7**). This is supported by earlier data showing that indomethacin-treated MyD88/TRIF DKO mice, deficient in TLR adapter molecules, had similar levels of colonic Tregs compared to vehicle mice, whereas indomethacin treated WT mice had a significantly greater number of both colonic $Foxp3^+$ and $ROR\gamma t^+$ Tregs compared to vehicle treated mice (**Fig. 5.2**). Similarly, there was no increase in colonic Tregs within the colons of antibiotic and indomethacin co-treated mice, compared to mice treated with indomethacin only. This may be due to the inability for LPS to signal via TLR4, or deficiency of microbiota, to induce IFNβ production.

Nakahashi-Oda. C, *et al* (2016) had demonstrated that culturing BMDCs with faecal content enhanced type 1 interferon genes significantly [6]. This demonstrated the importance of the gut microbiota in stimulating DCs to enhance proliferation of Tregs. It was already observed that the sterilely filtered caecum contents boosted $ROR\gamma t^+$ Tregs in a culture with $CD103^-$ MNPs (**Fig. 5.6**). Therefore, it was thought that potentially the sterilely filtered caecum content from indomethacin treated mice could increase type 1 interferon genes in BMDCs, and consequently boost Tregs within a co-culture. This could then potentially be used as DC immunotherapy. BMDCs were generated, sorted for $CD103^-$ DCs, then cultured with media, or sterilely filtered caecum contents from vehicle, indomethacin or indomethacin plus an EP4 agonist treated mice, and gene fold change measured by PCR. Interestingly there appeared to be a greater fold change of type 1 interferon genes in the BMDCs co-cultured with the sterilely filtered indomethacin caecum, compared to the media or, indomethacin plus an EP4 agonist filtrate, which suggested that the metabolites within this filtrate are able to boost regulatory functions of the BMDCs (**Fig. 5.8**).

Inhibition of endogenous $PGE_2$ expression resulted in increased colonic type 1 interferon gene expression, therefore to examine whether there was also increased signalling, phosphorylation of a downstream molecule STAT-1 was measured. As observed previously, indomethacin-treated mice had a greater proportion of colonic $Foxp3^+$ Tregs, and within this population, there was a significant increase in phosphorylated STAT-1 which was expected due to increased type 1 interferon signalling (**Fig. 5.9**). In addition to this, indomethacin treated mice had a greater proportion of colonic $CD11b^+$ cLP MNPs, and within this population there was also significantly greater phosphorylation of STAT-1, compared to vehicle treated mice. Type 1 interferons can act in either a paracrine or autocrine manner, hence can act on itself to continue to boost transcription of these genes [175].

It is evident that IFNβ production is increased when endogenous $PGE_2$ production is inhibited and this correlates with both colonic Foxp3 Treg numbers and $CD103^-$ cLP MNPs. Therefore, to finally confirm the importance of IFN signalling in Treg induction, IFNAR KO mice were used. WT indomethacin treated mice had a greater number of $Foxp3^+$ and $ROR\gamma t^+$ Tregs compared to indomethacin treated IFNAR mice (**Fig. 5.10**). This was similarly observed with $CD11b^+CD103^-$ cLP MNPs. Levels in the IFNAR KO mice were comparable to the vehicle group, impressing the importance of interferon signalling on $CD11b^+CD103^-$ MNPs (**Fig. 5.11**).

MyD88/TRIF DKO mice are resistant to transmitting TLR signals from bacterial products such as LPS and peptidoglycans, however they are still able to produce IFN-β which suggest there are MyD88 independent pathways to induce this [187]. There was not a significant increase in Tregs between the indomethacin and vehicle treated MyD88/TRIF DKO mice, but there was still a slight increase, which suggests that the microbial products signalling via TLR are important in induction of the $ROR\gamma t^+$ Tregs, but suggests that IFN-β signalling is also active and has a role (**Fig. 5.2**). This was similarly observed with the IFNAR KO mice, there was still a slight increase in $ROR\gamma t^+$ Tregs in the indomethacin treated IFNAR KO mice compared to the vehicle treated mice, demonstrating the importance of MyD88/TRIF signalling in combination with IFNAR signalling for induction of Tregs (**Fig. 5.11**). To potentially confirm that it is these two signalling pathways that are involved, MyD88/TRIF DKO mice could be treated with αIFNβ antibodies to block IFNβ signalling. After treating these mice with vehicle or indomethacin, Treg markers could be examined in colons to see whether there was still an increase in Tregs compared to vehicle-treated mice.

To confirm that indomethacin did not directly affect naïve T cell differentiation in the intestine, and that it was the inhibition of $PGE_2$ and resulting bacterial metabolites that affect Treg induction, naïve T cells were cultured with indomethacin. The indomethacin-treated cells had little reduction in Foxp3 expression compared to the media or vehicle treated group, whereas as observed in **chapter 1,** $PGE_2$ greatly reduced Foxp3 expression (**Fig. 5.12**). Indomethacin is readily absorbed 2 hours from when the drug was administered, and approximately 90 % of the dose is absorbed within 4 hours [188]. Therefore these data suggests that indomethacin does not directly affect naïve T cells, and the increased Tregs are due to inhibition of $PGE_2$ and resulting microbiota [188]. Additionally, indomethacin treatment has been shown to increase microbiota diversity, so there may an increased variety of metabolites that can potentially affect immune cell differentiation [183, 189].

## 5.5 Conclusion

These data suggested that type 1 interferon signalling via $CD103^-$ cLP MNPs also influenced $PGE_2$'s suppression of Tregs, which involved the gut microbiota. The next question was whether this knowledge could be used to reduce disease symptoms in a variety of different disease models to further confirm our findings.

# 6 The Role of $PGE_2$ on Intestinal Disease Models

## 6.1 Introduction

In **chapter 4, figure 4.6,** it was demonstrated that T cell specific EP4 receptor deletion did not affect the intestinal Foxp3 expressing Tregs, however there was a greater proportion of RORγt$^+$ expressing Tregs. RORγt$^+$ expressing Tregs are highly suppressive against pro-inflammatory cells such as Th1 and Th17 cells that may be present within the intestinal environment. Data from our group (unpublished) observed that caecum content from indomethacin treated mice had a positive effect on Foxp3 expression after being cultured with naïve T cells and I also demonstrated that it could influence CD103$^-$ mNP induction of Tregs (**Chapter 5, Fig**. **5.6**). It is known that intestinal microbiota can induce tolerogenic DCs, which can then induce Treg proliferation, therefore it would be useful to be able harness this by enhancing our understanding of how to influence gut microbiota to induce regulatory cells [67].

Disease models are important in research to confirm research hypothesises prior to further investigations and development of potential drugs for human use. They can also can confirm validity of previous research. Work discussed in the previous chapters demonstrated that an increase in colonic Tregs after inhibition of $PGE_2$ was prevented by use of broad-spectrum antibiotics, suggesting that the gut microbiota was involved in this process. Therefore, intestinal disease models were used to examine the role of $PGE_2$ in Tregs and colonic inflammation.

Boschetti, *et al* (2017) found that non-specific colonic inflammation enhanced lymphoid organ Treg differentiation and their suppressive ability, but negatively affected colonic DCs ability to convert naïve CD4$^+$ T cells into Tregs [190]. Inflammatory bowel diseases are T cell mediated disorders, involving both the innate and adaptive immune system. Thus, demonstrating the importance of understanding the role of the immune modulator prostaglandin $E_2$ within this system, its effect on both colonic Tregs and MNPs, and the potential protective role in colonic inflammation manipulation of this system could have.

Animal models of intestinal inflammation provide a multitude of information about the mucosal environment [191]. Dextran sulphate sodium (DSS) colitis is a severe, acute disease model which involves a sulphated polysaccharide disrupting the intestinal membrane after epithelial damage, enabling bacterial and intestinal antigens entry into the mucosal layer [191, 192]. This consequently stimulates a 'chronic' or 'acute' immune response. Research groups have historically used this disease model due to its low cost, simplicity to induce, and inducing an acute, chronic or relapsing model can be easily managed by changing the concentration of DSS administered [193]. Additionally, in the chronic stage of DSS colitis, the intestinal cell dysplasia observed is similar to what is observed in ulcerative colitis (UC) [192, 193].

Another intestinal disease that is used to examine intestinal inflammation is adoptive transfer colitis. Injection of naïve T cells can induce intestinal inflammation five to ten weeks from treatment, due to expansion of mature T cells [191]. Co-treatment with Tregs can be used to see the suppressive effect of these cells on pro-inflammatory T cell expansion. This is a milder disease model than DSS colitis as there is no chemical disruption to the intestinal membrane, therefore easier to see a smaller difference between treatment types. Fiona Powrie and her group led vital research into this, examining the role of Tregs in preventing T cell colitis development [190]. However the role Tregs have on in suppressing non-T cell mediated colitis is much less well known [190]. tTregs are developed to recognise and suppress a response to self-antigen, whereas pTregs suppress responses against both self-antigen, but also food and mucosal antigens [190]. pTregs are of highest importance within the mucosal environment, as deficiencies in pTregs result in intestinal inflammation. Naïve T cells were used in the T cell colitis disease model, as these could either convert to effector T cells or pTregs, influencing the intestinal inflammation.

Multiple groups have looked at the mechanisms for intestinal Treg induction and the benefits of microbial metabolites [45, 46, 52, 53, 190]. These are important due to the link between the intestinal microbiota and the immune system. Arpaia, *et al* (2013) demonstrated that administering SCFAs after antibiotic treatment resulted in a greater proportion of stable intestinal Tregs compared to mice treated with vehicle [194]. Additionally, other groups have found that various microbes such as *Clostridia* and *Bacteroides fragilis* can enhance intestinal Tregs and regulatory intestinal macrophages. Colonisation of the intestine by these regulatory cell inducing commensal microbiota were found to prevent DSS-induced colitis [38]. This demonstrates the great interest in this field, and how new information can benefit multiple groups.

Faecal transfer experiments are an exciting new field of research that can be used to determine the influence of gut microbiota on disease progression, which can then be utilised for development of treatment of intestinal inflammatory disorders. The work which I have previously discussed demonstrated that microbiota were involved in $PGE_2$'s suppression of intestinal Tregs and this was affected by antibiotic treatment. Xiao. *et al* (2017) demonstrated that just two days of treatment with the COX inhibitor indomethacin, altered the gut microbiota and there was a reduced proportion of *Bacteroidetes* and increased *Firmicutes* [195]. A transfer of faeces from these mice protected WT mice from indomethacin-induced injury and this was specifically dependent on the altered microbiota, as the sterile filtered solution did not protect against injury, and both COX and $PGE_2$ levels were similarly reduced in both groups treated with a high dose of indomethacin [195]. This is important, because current treatment for intestinal disease is maintaining remission by anti-inflammatory drugs, colectomy, or immunosuppressive drugs response [196, 197]. All which have potentially harmful side-effects, whereas altering the gut microbiota by faecal transplant or pre- or pro-biotics are cheaper, lower-risk and a more efficient way to enhance the 'good' gut microbiota that can likewise influence the anti-inflammatory response [196, 197]. It is also currently a successful method for treating *Clostridium difficile* infection [196]. Tian. Z, *et al* (2016) demonstrated the importance of faecal matter transplants by reducing disease activity in DSS-induced ulcerative colitis mice compared to those untreated by transferring via intra-rectal injection faeces from healthy donor mice [196].

## 6.2 Methods

To determine the role of $PGE_2$ signalling in disease induction, T cell specific EP4 KO ($LCK^{cre}EP4^{fl/fl}$) mice or control mice ($EP4^{fl/fl}$) were treated with 2 % DSS in drinking water for eight days, and disease severity scored daily (refer to **chapter 2**, **Section 2.9.1** for full details of scoring system). Mice were culled on day 8, colons removed and processed for flow cytometry to measure Foxp3 and IL-17 expression in live $CD45^+CD3^+CD4^+$ T cells. Data was analysed using Prism 6 software (GraphPad).

Additionally, to see whether inhibition of endogenous prostaglandin in WT mice would affect disease induction, WT (C57BL/6) mice were either treated with water only, 2 % DSS or 2 % DSS plus indomethacin in drinking water for 8 days. Disease severity and weight change was scored daily (refer to **chapter 2**, **Section 2.9.1** for full details of scoring system). Mice were culled on day 8, colons removed and processed for flow cytometry to measure Foxp3 expression in live $CD45^+CD3^+CD4^+$ T cells. Foxp3, IL-10 and pro-inflammatory genes such as IFNɣ and IL-17 were also measured using rtPCR from stored colon tissue.

To determine how EP4 KO T cells, preventing $PGE_2$ signalling, acted *in vivo,* naïve T cells (live $CD4^+CD25^-$ cells) were sorted from spleens either from $LCK^{cre}EP4^{fl/fl}$ or WT C57BL/6 mice then injected i.p. into $RAG^{-/-}$ mice to induce T cell colitis. Mice were monitored weekly until disease symptoms were observed, then monitored three times weekly (refer to **chapter 2**, **Section 2.9.2** for full details of scoring system). Mice were culled, colons extracted and stained for Treg markers and pro-inflammatory markers such as IL-17 and IFNɣ, then run on the LSR Fortessa.

It was thought that inhibition of $PGE_2$ not only affected colonic Tregs, but also involved the gut microbiota. Therefore, to examine whether indomethacin treatment affected the gut microbiota and resulted in a more protective phenotype, eight groups of donor mice were treated for five days with indomethacin (5 mg/kg/day) or vehicle (0.5 % ethanol), starting sequentially after each other. On day 5 of their individual treatment, the donor mice were culled, and caecum removed within a sterile hood. Caecum content was weighed and diluted 1:1 in the comparable volume of sterile cold PBS.

The solution was vortexed and then diluted 1:10 in sterile PBS and left at 4 $^{o}$C for the sediment to settle. This was done daily for fresh caecum content to be available for the recipient mice from day -2 to day 5, recipient mice were gavaged with the top layer of solution from the caecum content mixture. Recipient mice were given 2 % DSS colitis in their drinking water between day 0 and day 6, and monitored daily (refer to **chapter 2**, **Section 2.9.1** for full details of scoring system). On day 6, mice were culled and colons processed for flow cytometry.

### 6.2.1 Aims of chapter

- To determine whether inhibition of T cell specific $PGE_2$-EP4 signalling reduced disease severity in a DSS or T cell colitis disease model.
- To investigate whether inhibition of endogenous $PGE_2$ production reduced disease severity in a DSS-induced colitis disease model.
- To determine whether manipulation of the host gut microbiota by a caecum content transplant from COX inhibitor treated mice protected against a colitis disease model.

## 6.3 Results

### 6.3.1 EP4 signalling in T cells is dispensable in DSS-induced colitis

It was demonstrated in **chapter 4**, **figure 4.6**, that steady-state LCK$^{cre}$EP4$^{fl/fl}$ mice had a similar level of colonic Foxp3 Tregs compared to control mice, however expressed a greater proportion of RORγt$^{+}$. To see whether the LCK$^{cre}$EP4$^{fl/fl}$ mice were better at suppressing inflammatory disease than control mice, mice were treated for eight days with 2 % DSS in drinking water to induce DSS-colitis. Mice were culled, colons removed and Tregs were analysed on day eight (**Fig. 6.1a**). Disease severity was scored daily and mice were culled if severity reached 11 or above, or they lost more than 25 % of their initial body weight (**Fig. 6.1b**). The disease score was comparable between both groups of mice during the experimental period (**Fig. 6.1b**). Histograms demonstrating Foxp3 expression and flow cytometry dot plots of IL-17 expression in live CD45$^{+}$CD3$^{+}$CD4$^{+}$ cells from colons of EP4$^{fl/fl}$ and LCK$^{Cre}$EP4$^{fl/fl}$ mice induced with colitis are demonstrated in **Figure 6.1c**. There is no difference between colon lengths between the groups (**Fig. 6.1d**), and there was a similar percentage of Foxp3 and IL-17 detected too (**Fig. 6.1e, f**). In conclusion, LCK$^{cre}$EP4$^{fl/fl}$ mice were not better protected from colitis induction compared to control mice.

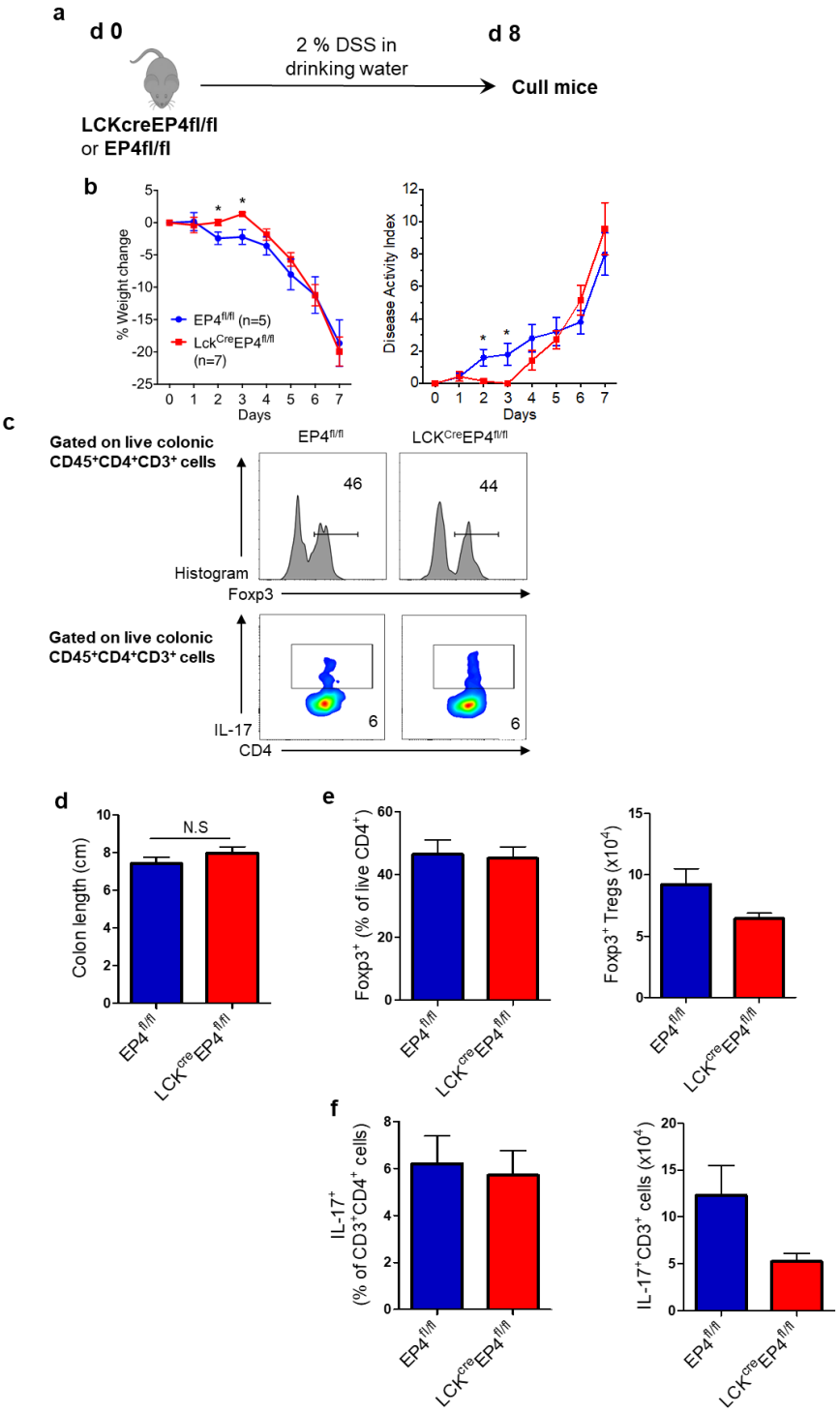


**Figure 6.1: The effect of T cell EP4 receptor deficiency on DSS-colitis:** *(a) Experimental timeline for inducing DSS-colitis in LCK$^{cre}$EP4$^{fl/fl}$ or WT mice. Mice were given 2 % DSS in drinking water for eight days. Day eight, mice were culled and tissues extracted and processed for staining. (b) Disease severity and percentage weight loss from starting weight was measured daily during the experiment. (c) Histograms demonstrate Foxp3 expression in live*

*$CD45^+CD4^+CD3^+$ colonic lamina propria cells, and flow cytometry dot plot demonstrating IL-17 expression in live $CD45^+CD4^+CD3^+$ colonic lamina propria cells. (d) After mice were culled, colons were extracted and measured, there was no difference between the average lengths of the colon between the two groups. (e) Graphs showing the percentage of $Foxp3^+$ and IL-$17^+$ T cells within live $CD4^+$ T cells in the colons of $LCK^{cre}EP4^{fl/fl}$ or WT ($EP4^{fl/fl}$) mice after DSS-colitis induction. (f) Graphs showing the percentage of $Foxp3^+$ and IL-$17^+$ T cells within live $CD4^+$ T cells in the mLNs of $LCK^{cre}EP4^{fl/fl}$ or WT ($EP4^{fl/fl}$) mice after DSS-colitis induction. Data shown as means ± SEM (error bars) n = 4 - 8. * p ≤ 0.05 by Mann-Whitney U test.*

### 6.3.2 Indomethacin treatment enhances both pro- and anti-inflammatory gene expression in DSS colitis mice

**Figure 6.1** demonstrated that inhibition of the $PGE_2$-T cell signalling pathway was not enough to protect mice from colitis induction, and in the previous chapter it was shown that the gut microbiota and $CD103^-CD11b^+$ DCs were also involved in Treg induction after inhibition of endogenous $PGE_2$. Therefore, DSS colitis was induced in C57BL/6 mice, and one group treated with indomethacin to inhibit endogenous $PGE_2$ production. One group was treated with water only as a control. Mice were culled on day eight and tissues processed for flow cytometry (**Fig. 6.2a**).

**Figure 6.2b** shows the flow cytometry dot plot of CD25 and Foxp3 expression in live colonic $CD45^+CD4^+CD3^+$ T cells. Mice induced with DSS colitis had a greater number and proportion of colonic $Foxp3^+$ Tregs, compared to control mice ($p \leq 0.05$) (**Fig. 6.2c**). However, levels were significantly enhanced when mice were also treated with indomethacin ($p \leq 0.05$) (**Fig. 6.2c**). *Foxp3* gene expression was likewise increased in mice treated with 2 % DSS compared to control mice ($p \leq 0.01$), and further enhanced in mice co-treated with indomethacin ($p \leq 0.05$) (**Fig. 6.2d**). This similarly correlated with levels of the Treg associated gene *il-10* ($p \leq 0.05$), and the pro-inflammatory cytokine *Ifn-γ* ($p \leq 0.05$), supporting the disease severity score which suggests that the increased number of Tregs within the colitis mice treated with indomethacin, are not completely protective against disease induction. Additionally, inhibition of $PGE_2$ only increased Foxp3 gene expression, it had little effect of *Il-10, Ifn-γ or IL-17* gene expression within the whole colon tissue (**Fig. 6.2c, d**). Treatment with indomethacin in combination with DSS did not reduce disease severity, suggesting that directly inhibiting endogenous $PGE_2$, although enhances colonic $ROR\gamma t^+$ Tregs, also has negative effects on other cells such as ILCs which negates the positive effect.

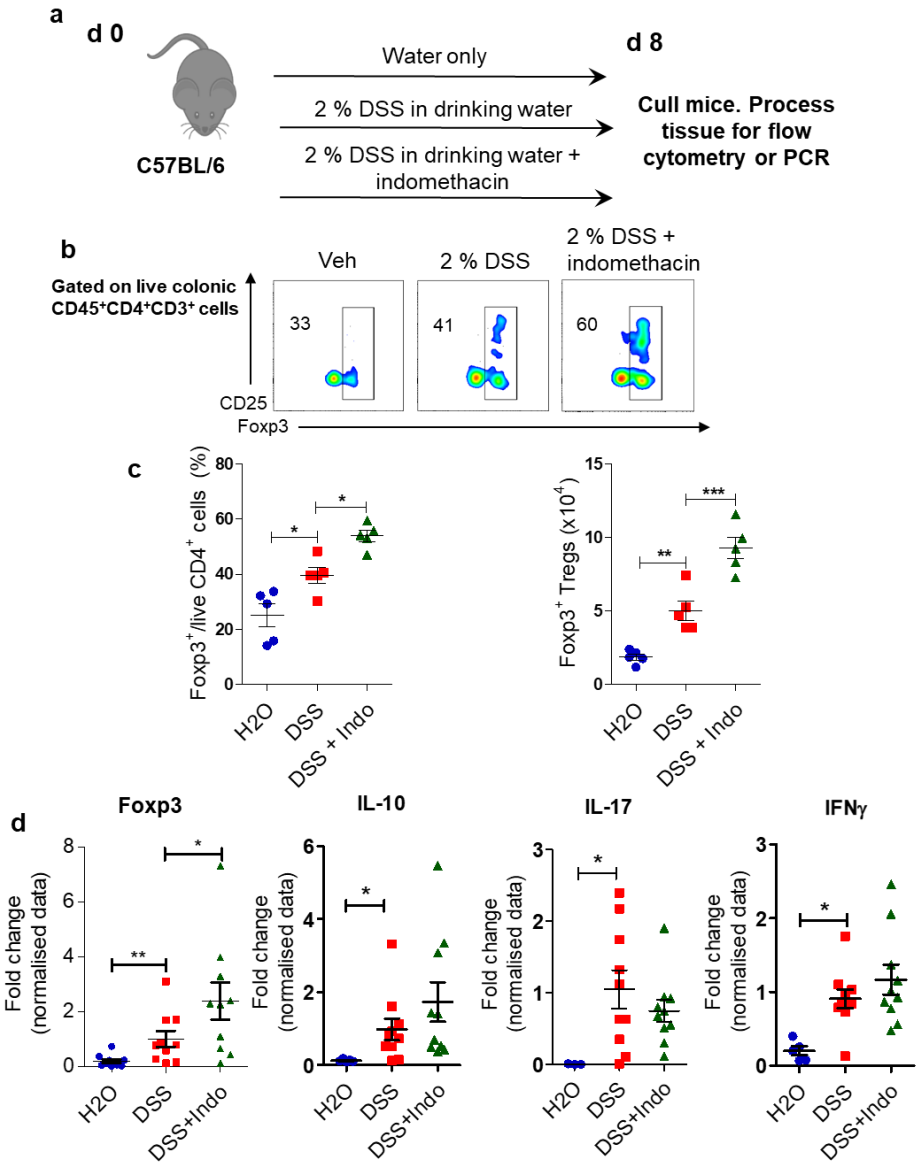


**Figure 6.2: The effect of endogenous $PGE_2$ inhibition on DSS induction of $Foxp3^+$ Tregs**. *(a) C57BL/6 mice were treated with water, 2 % DSS or 2 % DSS plus indomethacin in their drinking water for 8 days. On day 8, mice were culled and colons removed to be processed for flow cytometry. (b) Flow cytometry gating strategy for Foxp3 expression in live colonic $CD45^+CD4^+CD3^+$ cells. (c) Percentage and number of Foxp3 expressing T cells in colons of mice treated with water (control), DSS or DSS plus indomethacin. (d) Foxp3, Il-10, Ifn-ɣ and Il-17 gene expression from colons of mice treated with water (control), 2 % DSS and 2 % DSS plus indomethacin. Data shown as means ± SEM (error bars) n = 5-10. * $p \leq 0.05$, ** $p \leq 0.01$, $p \leq 0.001$ by Mann-Whitney U test.*

### 6.3.3 T cell colitis disease severity was reduced in mice injected with T cell specific EP4 KO cells

Inhibition of endogenous $PGE_2$ as a protective mechanism for a disease model is problematic because $PGE_2$ can also have a protective effect on other cell types such as ILC3s, therefore complete suppression of $PGE_2$ can damage the intestinal barrier, counteracting the positive effect of increased Tregs (as previously demonstrated). In **chapter 4, figure 4.6**, it was demonstrated that T cell specific EP4 KO mice had a greater proportion of colonic RORɣt$^+$ Tregs compared to control mice. Therefore, a T cell colitis disease model was induced by i.p injection of CD4$^+$CD25$^-$ T cells sorted from spleens of either WT mice or LCK$^{cre}$EP4$^{fl/fl}$ (EP4 KO) mice in RAG$^{-/-}$ mice on day 0 (**Fig. 6.3a**). This was to see whether disease severity would be reduced using EP4 KO T cells due to potentially a greater number of those cells converting to Tregs due to inhibition of $PGE_2$ signalling.

Mice injected with EP4 KO naïve T cells gained weight more easily until day 39, compared to mice injected with WT naïve T cells (**Fig. 6.3b**). In addition to this, only the mice injected with WT naïve T cells developed any mild disease symptoms (itchy eyes) during the experimental period (**Fig. 6.3b**). However, the colon length is similar between both groups (**Fig. 6.3c**). The flow cytometry plots for CD25, Foxp3 expression, and IL-17 and IFN-γ expression in Foxp3$^-$ T cells are demonstrated in **Figure 6.3d**. There are a significantly greater proportion of Foxp3$^+$ Tregs in the colons of RAG$^{-/-}$ mice administered with EP4 KO naïve T cells ($p \leq 0.01$), whereas those injected with WT naïve T cells had a higher proportion and number of Tconv cells ($p \leq 0.01$) (**Fig. 6.3e, f**). Mice injected with WT naïve T cells also had a greater number of IFNɣ$^+$ Tconv cells (thought to potentially be Th1 cells) ($p \leq 0.05$) (**Fig. 6.3g**). However, they had did have a smaller proportion of IL-17$^+$ Tconv cells (thought to potentially be Th17 cells) (**Fig. 6.3h**). These data suggest that RAG$^{-/-}$ mice injected with EP4 receptor KO live CD4$^+$CD25$^-$ T cells had reduced disease symptoms, and this may be due to the increased number of colonic Tregs.

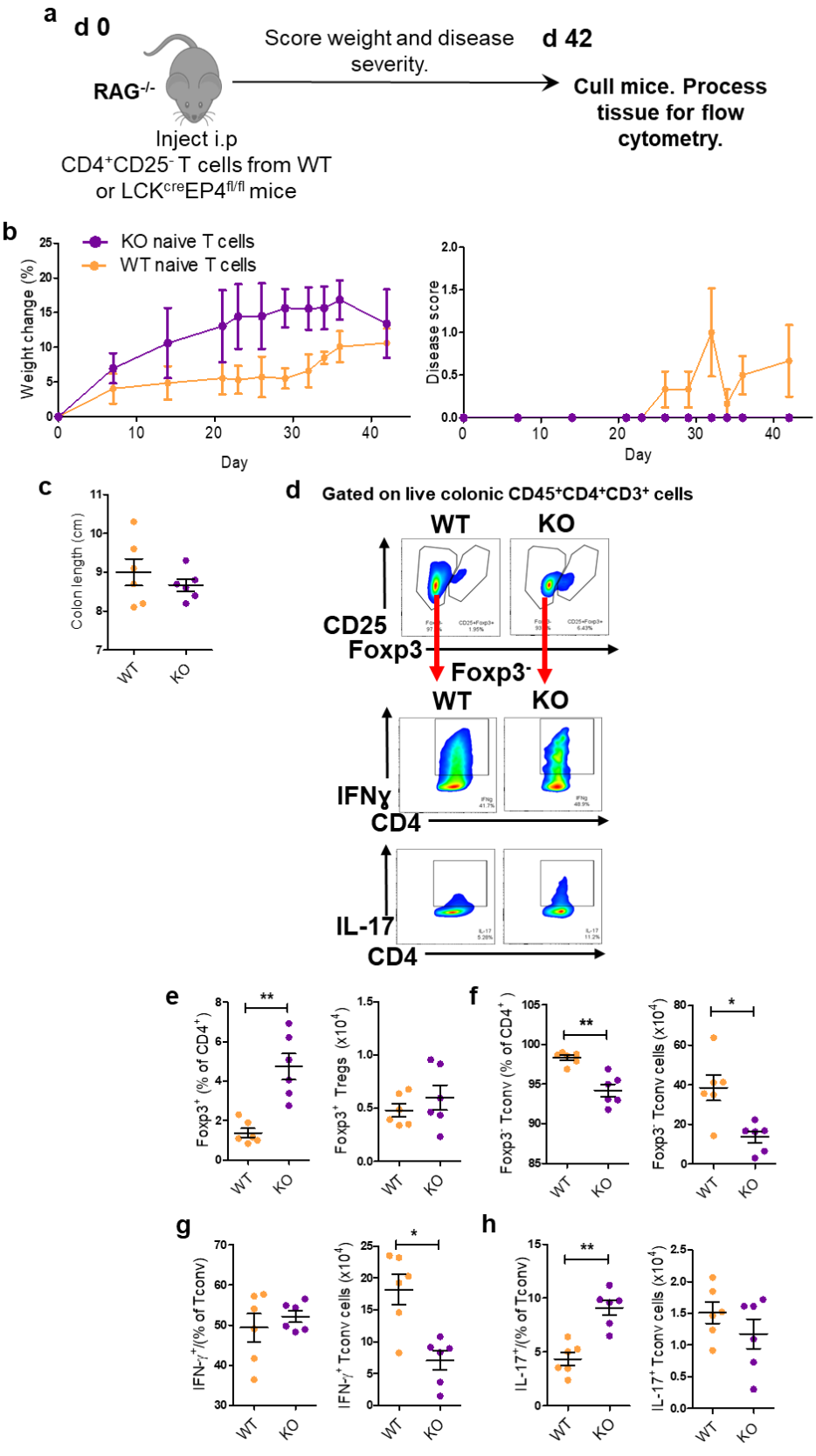
a
d 0
RAG-/-
Inject i.p
CD4+CD25- T cells from WT
or LCKcreEP4fl/fl mice
Score weight and disease severity.
d 42
Cull mice. Process tissue for flow cytometry.
b
KO naive T cells
WT naive T cells
Weight change (%)
Day
Disease score
Day
c
Colon length (cm)
WT
KO
d
Gated on live colonic CD45+CD4+CD3+ cells
WT
KO
CD25
Foxp3
Foxp3-
IFNγ
CD4
IL-17
CD4
e
Foxp3+ (% of CD4+)
Foxp3+ Tregs (x10^4)
f
Foxp3- Tconv (% of CD4+)
Foxp3- Tconv cells (x10^4)
g
IFN-γ+/(% of Tconv)
IFN-γ+ Tconv cells (x10^4)
h
IL-17+/(% of Tconv)
IL-17+ Tconv cells (x10^4)

**Figure 6.3: The effect of T cell specific EP4 KO on T cell colitis induction.** (*a) RAG$^{-/-}$ mice were injected with sorted CD4$^{+}$CD25$^{-}$ T cells from either WT or EP4 KO mouse spleens on day 0. Weight and disease severity was scored weekly until disease symptoms were apparent, then mice were scored three times a week until day 42. On day 42, mice were culled, colons removed and tissues processed for flow cytometry. (b) Percentage weight change from original weight and disease score over 42 days. (c) Colon length of colons from mice injected with either WT or EP4 KO T cells. (d) Flow cytometry plots of Foxp3 expression in live colonic CD45$^{+}$CD4$^{+}$CD3$^{+}$ cells from mice injected with WT or EP4 KO T cells, and pro-inflammatory cytokines IFNɣ and IL-17 expression in live colonic CD45$^{+}$CD4$^{+}$CD3$^{+}$Foxp3$^{-}$ cells. (e) Percentage and number of Foxp3 expressing colonic Tregs in mice injected with naïve T cells from WT or EP4 KO mice. (f) Percentage and number of Foxp3$^{-}$ expressing colonic Tconv cells in mice injected with naïve T cells from WT or EP4 KO mice. (g) Percentage and number of IFNɣ$^{+}$ expressing colonic Tconv cells in mice injected with naïve T cells from WT or EP4 KO mice. (h) Percentage and number of IL-17$^{+}$ expressing colonic Tconv cells in mice injected with naïve T cells from WT or EP4 KO mice. Data shown as means ± SEM (error bars) n = 6. * p ≤ 0.05, ** p ≤ 0.01 by Mann-Whitney U test.*

### 6.3.4 $PGE_2$ exacerbates intestinal inflammation through driving microbial shifts.

It was observed that inhibition of $PGE_2$ resulted in increased colonic lamina propria Tregs, which are known to suppress inflammatory markers and co-treatment with antibiotics prevented this, suggesting that gut microbiota are involved in $PGE_2$'s modulation of Tregs. Therefore, a DSS colitis model was carried out using faecal transplants from either vehicle- or indomethacin-treated mice, to see whether microbes and metabolites from treated mice could influence disease progression.

Eight groups of donor mice were treated with indomethacin (5 mg/kg/day) or vehicle (0.5 % ethanol) for five days sequentially. On day 5 of the respective group's treatment, donor mice were culled, caecum extracted and contents removed under sterile conditions and diluted 10 X in sterile PBS. Contents were vortexed, left for the solid particles to settle before the solution was gavaged into recipient mice from day -2 to day 5. Recipient mice were given 2 % DSS in drinking water from day 0 until day 6, and scored and weighed daily (**Fig. 6.4a**). Recipient mice gavaged for eight days with fresh and sterile caecum solution from indomethacin-treated mice were better protected against DSS colitis induction compared to mice gavaged with filtrate from vehicle-treated mice (**Fig. 6.4b**). They had less severe weight loss, and a better disease score over the seven-day colitis disease period (**Fig. 6.4b**). Colon length was similar between the two groups (**Fig. 6.4c**). Flow cytometry dot plots of CD4 and Foxp3 expression in live colonic lamina propria $CD45^+CD3^+$ T cells, and IL-17 and IFNɣ expression in live colonic lamina propria $CD45^+CD4^+CD3^+Foxp3^-$ Tconv cells is demonstrated in **figure 6.4d**. Recipient mice gavaged with caecum solution from indomethacin-treated mice had a greater proportion of colonic Foxp3 expressing T cells ($p \leq 0.01$), and likewise had smaller proportion of $Foxp3^-$ Tconv cells ($p \leq 0.01$) (**Fig. 6.4d, e**). These mice also had a reduced number of colonic IFNɣ expressing Tconv cells thought to be pro-inflammatory Th1 cells ($p = 0.065$) (**Fig. 6.4e**).

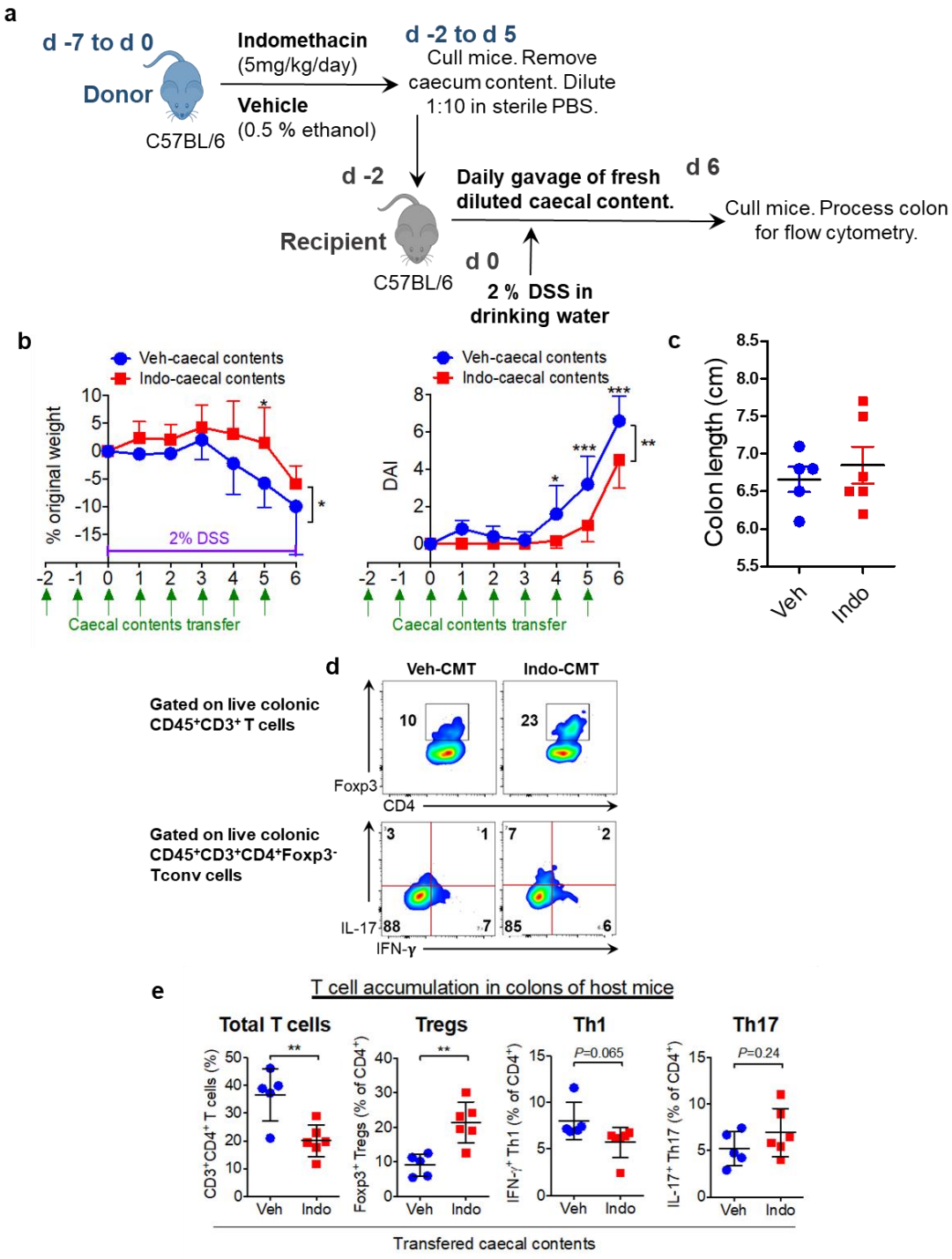
a
d -7 to d 0
Donor
C57BL/6
Indomethacin
(5mg/kg/day)
Vehicle
(0.5 % ethanol)
d -2 to d 5
Cull mice. Remove caecum content. Dilute 1:10 in sterile PBS.
d -2
Recipient
C57BL/6
Daily gavage of fresh diluted caecal content.
d 6
Cull mice. Process colon for flow cytometry.
d 0
2 % DSS in drinking water
b
Veh-caecal contents
Indo-caecal contents
% original weight
2% DSS
Caecal contents transfer
DAI
c
Colon length (cm)
Veh
Indo
d
Veh-CMT
Indo-CMT
Gated on live colonic CD45+CD3+ T cells
Foxp3
CD4
Gated on live colonic CD45+CD3+CD4+Foxp3- Tconv cells
IL-17
IFN-γ
e
T cell accumulation in colons of host mice
Total T cells
Tregs
Th1
Th17
P=0.065
P=0.24
Transfered caecal contents

**Figure 6.4: The effect of an altered gut microbiota on colitis development.** *(a) Eight groups of donor mice were treated with either indomethacin (5 mg/kg/day) or vehicle (0.5 % ethanol) for five days starting in a sequential manner. Mice were culled and caecum content removed under sterile conditions. Caecum content was diluted 1:10 with sterile PBC, then gavaged daily into recipient mice. Recipient mice were gavaged with fresh caecum content from day -2 until day 6, and treated with 2 % DSS colitis in drinking water from day 0 until day 6. (b) Percentage weight change from original weight and disease score over an eight day experimental period. (c) Colon length of DSS colitis mice gavaged with caecum content from indomethacin or vehicle treated mice. (d) Flow cytometry dot plots of Foxp3 expression in colonic live $CD45^+CD4^+CD3^+$ cells and pro-inflammatory cytokines IFNɣ and IL-17 expression in live colonic $CD45^+CD4^+CD3^+Foxp3^-$ cells from DSS colitis mice gavaged with caecum content from indomethacin or vehicle treated mice. (e) Percentage and number of Foxp3 expressing colonic Tregs in mice injected with naïve T cells from WT or EP4 KO mice. (f) Percentage and number of $Foxp3^-$ expressing colonic Tconv cells in mice injected with naïve T cells from WT or EP4 KO mice. (g) Percentage and number of $IL\text{-}17^+$ expressing colonic Tconv cells in mice injected with naïve T cells from WT or EP4 KO mice. (h) Percentage and number of $IFNɣ^+$ expressing colonic Tconv cells in mice injected with naïve T cells from WT or EP4 KO mice. Data shown as means ± SEM (error bars) n = 5-6. * p ≤ 0.05, ** p ≤ 0.01 by Mann-Whitney U test.*

## 6.4 Discussion

At this point, I have shown that EP4 KO T cells induce less severe colitis in RAG mice compared to WT T cells, and pre-treating mice with caecum content from indomethacin-treated mice was more protective against DSS colitis induction compared to mice pre-treated with caecum content from vehicle-treated mice.

It was demonstrated in **chapter 4, figure 4.6,** that T cell specific EP4 conditional KO mice had a similar number of Foxp3 expressing colonic cells compared to WT mice, however within this population there was a greater number of RORɣt$^{+}$ Tregs. These Tregs are known to be highly suppressive within the potentially inflammatory environment of the colon due to increased levels of mTGFβ and colonic homing markers CD62L, therefore to determine whether these mice were more protected from disease, DSS colitis was induced in either WT or EP4 KO mice [46]. WT mice had greater weight loss and disease activity score compared to EP4 KO mice over the first few days, however at the end of the disease progression, there was no difference between the two groups with regard to weight change, disease score, regulatory Foxp3$^{+}$ Tregs or pro-inflammatory IL-17 Tconv cells (**Fig. 6.1**). Blocking $PGE_2$ signalling via inhibition of EP4 signalling was not enough to protect mice from colitis induction, therefore colitis induction was repeated again with additional indomethacin treatment which was previously shown to enhance colonic RORɣt$^{+}$Foxp3$^{+}$ Tregs in **chapters 4** and **chapter 5**.

Mice treated with indomethacin in addition to colitis induction had a greater number and proportion of Foxp3$^{+}$ Tregs compared to vehicle and DSS-colitis only mice (**Fig. 6.2**). However, these increased Tregs did not provide increased protection again disease (data unpublished), because indomethacin and DSS co-treated mice had a more severe disease phenotype than even DSS-alone. This is due to indomethacin having a negative effect on the colonic environment due to gastrointestinal damage [35, 198-200]. This is confirmed by the PCR results which demonstrated that although colonic tissue from indomethacin-treated colitis mice had a greater fold change of *Foxp3* and *Il-10* expression, there was also an increase in the pro-inflammatory markers *Ifn-ɣ* and *Il-17* (**Fig. 6.2**).

NSAIDs are known to have long-term benefits, such as reducing the risk of certain diseases such as Alzheimer's, however side-effects are also associated with their use such as gastrointestinal damage and increasing severity of IBD [35, 137, 157, 201]. Similarly using specific prostaglandin synthase inhibitors are totally inefficient, as AA can be redirected to another prostaglandin, resulting in other adverse effects [83]. Therefore, if the complex role of $PGE_2$ cannot be fully understood or harnessed directly, then the role that it has on microbiota and benefits of this can be used more efficiently to regulate the colonic immune system.

Due to our previous work in **chapter 4**, **figure 4.6**, demonstrating the increased number of colonic RORɣt$^+$ Tregs T cell specific EP4 KO mice had compared to control mice, a T cell colitis model using naïve T cell from these two genotypes of mice was used to see the effect of these cells on disease induction. Some groups suggest that DSS colitis cannot be directly compared to human IBD as DSS colitis is a result of massive immediate damage to the epithelial layer and is a result of mixed pro-inflammatory T cell involvement, whereas IBD normally involves polarised T cells and slower disease development therefore a milder disease course using T cell colitis was also observed [191]. It was demonstrated that inhibition of $PGE_2$ increased type 1 interferon signalling in Tregs and DCs, similar to the increased levels of RORɣt expression that was observed in the Foxp3$^+$ Tregs.

It was noted that mice injected with naïve T cells from T cell specific EP4 KO mice gained more weight during the disease period compared to the WT mice, and similarly there were a greater number of colonic Foxp3 Tregs and fewer IFNɣ$^+$ Tconv cells (thought to be a Th1 phenotype) (**Fig. 6.3**). However, there are a greater number of IL-17$^+$ Tconv cells (thought to have a Th17 phenotype). The intestine favours pTreg generation, of which 40 – 60 % RORɣt, therefore as EP4 KO mice at a steady-state condition had a greater proportion of colonic RORɣt$^+$ Tregs compared to WT mice a greater proportion of these naïve T cells may have converted to the anti-inflammatory Tregs within the colonic environment and consequently allowed better weight gain compared to mice injected with WT cells [55, 60].

Yang *et al* (2016), demonstrated the importance of RORɣt$^{+}$ Tregs by an adoptive transfer of a suboptimal ratio of Foxp3$^{+}$ or RORɣT$^{+}$Foxp3$^{+}$ Tregs to naïve T cells in mice, to observe the difference in suppressive ability [55]. Mice given RORɣt$^{+}$Foxp3$^{+}$ Tregs had better protection compared to RORɣt$^{-}$Foxp3$^{+}$ Tregs [55]. The increased type 1 interferon signalling also enhanced Treg suppressive ability, potentially reducing the T cell colitis disease severity that was observed [76]. Additionally, interferon-STAT-1 signalling is known to enhance Treg differentiation which was shown to be enhanced in colonic Tregs and DCs, whereas interferon-STAT-4 signalling enhanced Th1 cell induction, demonstrating the importance of the signalling pathway. Future work should examine STAT molecules within the stored colonic tissue and cells to see whether STAT-4 is similarly altered in intestinal inflammation [76]. This correlates with what was observed with our T cell colitis model, where naïve T cells from EP4 KO mice were expected to have a greater level of RORɣt expression. This is also potentially why there was a greater number of IL-17 expressing cells within the colon, as well as Foxp3 Tregs as Foxp3$^{+}$RORɣt$^{+}$ T cells have been described as intermediate cells which can further differentiate into mature Foxp3$^{+}$ Tregs or RORɣt$^{+}$ T conv cells. This experiment should be repeated while also weighing WT mice as controls during the experimental process to compare natural weight gain. However, this initial preliminary experiment demonstrated that T cells unable to transmit the $PGE_2$ signal via EP4, resulted in a better disease course and were more protective than WT T cells, and this correlated with colonic Treg numbers.

As demonstrated in **chapter 5, figure 5.6,** and data from our group (unpublished), sterile filtered caecum content from indomethacin-treated mice enhanced Tregs and boost CD103$^{-}$ MNPs ability to induce Tregs *in vitro*. Additionally Xiao, X. *et al* (2017), saw that indomethacin treatment altered murine gut microbiota, which resulted in an increase in *Firmicutes* whereas there is a reduction in *Bacteroidetes* [195]. The importance of the gut microbiota was evident because both $PGE_2$ and COX levels were similarly reduced in indomethacin-injury induced mice, thus suggesting that inhibition of $PGE_2$ in the donor mice resulted in more protective microbiota, and also pharmacodynamics were unaffected [195].

Use of indomethacin altered the gut microbiota resulting in a more protective phenotype, therefore I wanted to determine whether this protected mice against DSS colitis induction. Caecum content from donor indomethacin-treated mice was transferred two days prior to colitis induction until day 7 to potentially alter the gut microbiota of recipient mice and protect against disease. Xiao *et al* (2017), saw mice treated with caecum content from indomethacin treated mice had better protection against indomethacin-induced disease, and similarly were protected from DSS colitis disease progression [195]. They did not observe any protective benefit when the caecum content was filtered so only microbial metabolites remained, therefore in the DSS colitis disease model, caecum content was not filtered, yet for ease of gavage the solid particles were allowed to settle before drawing up the clear solution. However, our group has previously observed that sterilely filtered caecum content from indomethacin-treated mice were able to enhance Foxp3 expression in naïve T cells (data unpublished) and also in a naïve T cell-CD103$^-$ DC co-culture (**chapter 5, figure 5.6**).

Similar to what was observed with the T cell colitis disease model, colitis mice gavaged with the caecum contents from indomethacin-treated mice lost weight at a slower rate than mice gavaged with caecum content from vehicle-treated mice (**Fig. 6.4**). Similarly, they also had a greater proportion of Foxp3$^+$ Tregs, and IFNɣ$^+$ Tconv cells compared to vehicle-caecum content treated mice, whereas the vehicle-caecum treated mice had a greater number of pro-inflammatory Foxp3$^-$ Tconv, and IL-17$^+$ Tconv cells. A comparable result was seen in Xiao's paper, where vehicle-treated mice had a greater proportion of inflammatory markers such as IFN-β and TNF-α [195]. However Tian, Z, *et al* (2016) observed an improvement in disease activity score in DSS-induced ulcerative colitis BALB/c mice after faecal matter transfer compared to those not given faecal matter transfer, however they still saw a significant weight loss in C57BL/6 mice after a faecal matter transfer similar those without treatment suggesting that mouse background or housing facilities could influence this [196]. However, I saw an improvement in disease activity scores in C57BL/6 mice given a faecal matter transfer from indomethacin treated mice, compared to transfer from vehicle treated mice. Foxp3$^+$ Tregs have been shown to enhance divergence of gut microbiota, and regulation of IgA production, all which is important for intestinal homeostasis [167].

Multiple groups have demonstrated that commensal microbiota are important in maintaining type 1 interferon expression in the mucosal tissue, which regulates the immune response for various challenges [76]. And conversely, deficiency in *Ifnar1* affected the commensal microbiota diversity, demonstrating the importance of interferon signalling in the intestinal immune response [76]. Thus, suggesting that altering the gut microbiota of the DSS colitis mice with caecum content from indomethacin-treated mice could boost interferon signalling and consequently enhance intestinal Tregs to protect from colitis.

This indicates that other methods to boost intestinal Tregs, rather than directly inhibiting $PGE_2$, could be used even more efficiently through understanding of the mechanism of $PGE_2$ suppression of Tregs.

It should be considered further whether inhibition of $PGE_2$ is directly affecting Tregs which can consequently affect the gut microbiota, or whether it affects the gut microbiota which can consequently influence Treg expression [167].

## 6.5 Conclusion

The caecum contents from indomethacin-treated mice had a protective effect on DSS colitis mice, which was expected from previous results. Additionally, EP4 KO T cells induced a milder version of T cell colitis, potentially due to the increased colonic Tregs that mature due to inhibition of $PGE_2$ signalling.

# 7 Discussion

## 7.1 Rationale for study

Prostaglandin $E_2$ ($PGE_2$) is a ubiquitously expressed molecule that is implicated in multiple biological processes such as fertility, gastric mucosal integrity, and the immune response. $PGE_2$ is involved in the key signs of inflammation; rubor (redness), calor (heat), tumor (swelling) and dolor (pain), and recent work has demonstrated that it can have both pro- and anti-inflammatory properties depending on the EP receptors activated and concentrations of $PGE_2$ present at the site [83].

Self-reactive T helper cells (Th) cells (i.e. Th1 and Th17) can induce autoimmune and autoinflammation. These inflammatory cells can be suppressed by regulatory T cells (Tregs), which express the transcription factor Foxp3. The equilibrium between Th1/Th17 cells and Tregs is important to not only prevent the development of autoimmunity, but also the induction of cancer for example, due to Tregs preventing T helper cells in recognition and aiding destruction of damaged cells. It has been demonstrated that $PGE_2$ aids Th1 and Th17 cell induction and proliferation and therefore supports inflammation development. However, the role of $PGE_2$ in Treg development and function is not as clear [91, 102]. The work presented here aimed to test the hypothesis that $PGE_2$ signalling regulates Treg Foxp3 expression, which consequently affects development and function, facilitating intestinal inflammation.

## 7.2 *In vitro* work – direct actions of $PGE_2$ on Tregs

$PGE_2$, acting via the EP2 and EP4 receptors, has previously been indicated to increase Th1 cell differentiation and expand the Th17 population [102, 156]. As helper T cells are controlled by regulatory T (Treg) cells, I wondered whether $PGE_2$ promotes inflammatory response by also affecting Tregs. I found that in an *in vitro* culture system, $PGE_2$ negatively affects Foxp3 expression in Tregs, and this is mediated by the receptor subtypes EP2 and EP4 (**chapter 3, Fig. 3.1, 3.4**). EP2 and EP4 have previously been shown as key $PGE_2$ receptors in regard to effector Th1 cell differentiation, and Th17 cell expansion [91, 102]. Using agonists and antagonists to examine the signalling pathways involved, I further found that this was due to their cAMP/PKA and PI3K pathways (**chapter 3. Fig. 3.4, 3.5**). These findings were in agreement with previous reports that discuss the transcription factor CREB as an important molecule in balancing effector Th17 cells and regulatory Tregs, negatively influencing $Foxp3^+$ Treg survival [33, 34, 159].

The cAMP response element binding protein (CREB) transcription factor, a member of the basic leucine zipper superfamily, has previously been thought to bind to the promoter and conserved non-coding sequence 2 (CNS 2) within the *Foxp3* gene, and increase expression of this gene [34]. Moreover, a recent paper showed that activation of CREB promoted Th17 differentiation *both in vitro* and *in vivo*, and reduced survival and expansion of $Foxp3^+$ Tregs [33, 34]. cAMP-responsive element modulator (CREM) is also a member of the basic leucine zipper superfamily, and the alpha isoform was found to enhance IL-17 protein (a Th17 family cytokine) production through binding of the gene promoter region [33, 34]. Wang. X, *et al* (2017) used a T cell colitis model to determine the importance of CREB, activated after $PGE_2$ – EP4 signalling, and observed reduced inflammatory cytokine production from CREB deficient $CD4^+$ T cells and a better disease activity score, compared to WT $CD4^+$ T cells [34]. These findings together suggest that the reduction in $Foxp3^+$ Tregs is due to $PGE_2$ activating the EP2 and EP4 receptors, and signalling via the cAMP-CREB pathway.

Although some groups have shown a positive link between $PGE_2$ and $Foxp3^+$ Tregs, Baratelli, F. *et al* (2005) detected an upregulation in Foxp3 expression in human $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T cells after culturing cells with supernatant from COX-2 overexpressing tumour supernatant [202]. However, they did observe a reduction in CD25 levels, similar to the data presented in this thesis (**chapter 3, Fig. 3.1, Fig. 3.3**). Sharma, S. *et al* (2005) also observed that $PGE_2$ enhanced Treg Foxp3 levels in COX-2 overexpressing tumour environments, and inhibition of COX-2 prevented this [153]. The EP4 receptor was shown to be vital in this process, and deficiency in this receptor reduced Treg Foxp3 expression, and was completely prevented by co-suppression of the EP2 receptor. The importance of these receptors was observed in my studies (**chapter 3, Fig. 3.4**). Increased Foxp3 expression has been detected in tumour microenvironments when the enzyme COX-2 is overexpressed [129, 158, 203]. However within tumour tissues, cells may be secreting various other molecules such as chemokines like CCL28, which can recruit Tregs via stimulation of the chemokine receptor CCR10, also expression of other tumour derived factors can cause Treg expansion, such as TGF-β [204]. Therefore, these other factors should be considered within the tumour environment.

Gene microarrays carried out by our group (unpublished data) suggested that $PGE_2$ disrupted the TGF-β signalling pathway through suppression of *Tgfbr1* and increasing expression of *Smad6* and *Smad7*, both of which have an inhibitory effect of TGF-β signalling (data not shown). Additionally, there was reduced phosphorylated SMAD2 and phosphorylated SMAD3 levels, downstream of the TGF-β receptor, in Tregs present within the $PGE_2$ treated cell population compared to vehicle cultured cells. This suggests that $PGE_2$ is negatively affecting the TGF-β signalling pathway. This can also influence Foxp3 expression through disrupting the TGF-β signalling pathway. This *in vitro* work provided useful evidence supporting the hypothesis that $PGE_2$ negatively effects Foxp3 expression at a range of TGF-β concentrations, and addition to the cell culture 24 hours after initial plating removed any effect due to cell viability. However, it was necessary to also confirm this *in vivo* prior to further investigations into $PGE_2$'s mechanism of action on $Foxp3^+$ Tregs.

## 7.3 *In vivo* studies

I also investigated the roles of endogenous $PGE_2$ on tissue resident Tregs *in vivo*, and found that $PGE_2$ suppresses intestinal $Foxp3^+$ Tregs, especially $ROR\gamma t^+Foxp3^+$ Tregs via the EP4 receptor. Further supporting the role of $PGE_2$ in this response, an increase in $ROR\gamma t^+$ Tregs was also observed in colons of T cell specific EP4 knock-out mice compared to WT mice, demonstrating that suppressing the $PGE_2$ signalling pathway influences RORγt expression in Foxp3 expressing intestinal Tregs (**chapter 4, Fig. 4.6**). Data obtained from *in vitro* experiments demonstrated that Foxp3 expression was affected in iTreg cell cultures treated with $PGE_2$, whereas nTreg activation status was affected ($CD25^+Foxp3^+$) however total *Foxp3* gene expression was unaffected (**chapter 3, Fig. 3.3**). Inhibition of $PGE_2$ production by NSAID treatment resulted in increased Foxp3 expression in Tregs present within the mesenteric lymph nodes, spleen and colon, however the proportion and number of $Foxp3^-$ Tconv cells were unaffected, suggesting that the increase in Tregs was not due to inflammation (**chapter 4, Fig. 4.1**). Use of NSAIDs is known to increase the risk of colonic inflammation, due to deleterious changes in mucosal hydrophobicity and epithelial pH, erosion of the gastroduodenal mucosa and increases in intestinal permeability.

Wang. X, *et al* (2017) used CREB knock-out mice to observe the effect of inhibition of the $PGE_2$ – EP4 signalling pathway on Th17 and Foxp3$^+$ Treg development *in vivo* [34]. They observed that preventing CREB signalling resulted in increased intestinal Foxp3$^+$ Tregs while reducing Th17 cell numbers, and was protective in a colitis disease model [34]. Thus, suggesting that $PGE_2$ signalling via EP4 within the intestine, inducing CREB signalling via cAMP, negatively effects Foxp3$^+$ Treg levels, as inhibition of $PGE_2$ using a NSAID resulted in increased intestinal Tregs.

Within the colonic Foxp3$^+$ Tregs, inhibition of $PGE_2$ also resulted in an increased proportion and number of RORɣt$^+$ expressing Tregs, whereas RORɣt$^+$Foxp3$^-$ Tconv cells were unaffected (**chapter 4, Fig. 4.2**). However, there was no similar increase observed within Tregs from spleens or mLNs. Intestinal RORɣt$^+$Foxp3$^+$ Tregs are a sub-population of peripheral derived Tregs (pTregs) which represent approximately 50 % of this population, vitally important within this environment due to their stable suppressive ability within an inflammatory environment and due to significant demethylation at Treg signature genes, such as *Foxp3* and *Ctla-4* [55, 62, 167]. High levels of membrane-bound TGF-β (mTGF-β) and CD62L result in this Treg subset having a better ability to respond to inflammatory signals [55, 167]. The importance of this Treg subtype is well known, and Yang *et al* (2016), demonstrated their ability to suppress inflammation by injecting suboptimal numbers of either RORɣt$^+$ or RORɣt$^-$ Tregs alongside naïve T cells to determine their effect [55]. RORɣt$^+$Foxp3$^+$ Tregs had an increased suppressive compared to RORɣt$^-$Foxp3$^+$ Tregs in a T cell colitis model [55, 62].

IL-6 and TGF-β signalling are both involved in induction of RORɣt expression, which can activate molecules essential for Th17 differentiation [205]. It was thought that cells co-expressing Foxp3 and RORɣt can become inflammatory due to the balance of TGF-β and inflammatory cytokines, and produce the Th17 associated cytokine IL-17 after IL-6 treatment due to 'plasticity' of the Tregs, which was considered after fate-mapping in mice indicated that a number of effector T cells come from a Treg lineage [62, 205]. However, it was more recently demonstrated that the Foxp3$^+$RORɣt$^+$ co-expressing Tregs have a stable lineage, because although Foxp3$^+$RORɣt$^+$ Tregs express both Treg and Th17 signature genes, transcriptomic and epigenetic profiling detected that their similarity was closer to Foxp3$^+$RORɣt$^-$ Tregs rather than Foxp3$^-$ RORɣt$^+$ effector T cells [62].

Foxp3$^+$ Tregs co-expression of Th1/ Th2/ Th17 transcription factors; T-bet, GATA-3 and RORɣt, is beneficial for the cells as it boosts the Tregs suppressive ability against the respective T effector cells [25]. It is thought that the shared receptor expression increases the efficiency of homing to the inflammatory environment, and helps maintain Treg functionality during a specific effector T cell inflammatory response [25, 180]. An adoptive transfer model was used to determine the *in vivo* stability of these cell populations [62]. Approximately 90 % of Foxp3$^+$ Tregs maintained Foxp3 expression after transfer, and around 80 % of Foxp3$^+$RORɣt$^+$ co-expressing Tregs maintained Foxp3 expression, and RORɣt expression was unaffected, however this was in the intestine, in other organs, such as the spleen, RORɣt levels were reduced [62]. Additionally, colonic Foxp3$^+$ Tregs significantly enhanced RORɣt expression when re-isolated from colonic tissue, however adoptively transferred RORɣt$^+$ T effector cells saw only a slight increase in Foxp3 expression, demonstrating that the dual RORɣt and Foxp3 expressing cells developed from FoxP3$^+$ Tregs [62] This demonstrated the stability of this regulatory cell lineage which can be maintained within the intestine, thus understanding the mechanisms behind the induction and maintenance of this cell population could potentially be beneficial for further research into inflammatory intestinal conditions.

For future work, it would be useful to use an antibody against $PGE_2$ for further confirmation of the direct role of $PGE_2$. As previously mentioned, NSAIDs and COX inhibitors can have mixed results as treatment for inflammation, especially within the colon, therefore it may be more important to understand the mechanism by which $PGE_2$ suppresses intestinal RORɣt$^+$ Tregs. Manipulation of this mechanism could then be used to develop drugs to more effectively reduce colonic inflammation.

## 7.4 Intestinal immunity

The intestine is a highly variable environment with a large interface. In human adults, the small intestine is on average 6 metres long, while the large intestine is approximately 1.5 metres long [206]. This ensures that the immune system and gut microbiota are in close contact, thus maintaining homeostasis is key to prevent unnecessary colonic inflammation. Aberrant expression of the COX enzymes, notably COX-2 which is greatly upregulated in an inflammatory environment, increases $PGE_2$ expression within tissues, potentially resulting in cancer development [84, 129]. This demonstrates the potential harmful effect that overexpression of $PGE_2$ has within this environment, and the importance of understanding the effect of suppressing levels of this molecule.

Increased levels of prostaglandins have been observed in various conditions such as pre-malignant and malignant colorectal tumours, demonstrating the importance in understanding both the benefits and issues with suppression of this molecule [84, 129]. Loughry, R. W (1983); Chan, A. T (2007); Levin, B (2006); Chan, A. T (2014); Sørensen. H, T (2015); are just a few examples of the growing number of groups that have observed a link between long-term aspirin use and a reduced risk of colorectal cancer [122-126]. Groups have shown that hydroxyprostaglandin dehydrogenase 15 (15-PGDH) is vital in the antagonism of prostaglandin-endoperoxide synthase 2 (PTGS2), encoding COX-2, during cancer development [125].

COX enzymes and their consequent prostaglandin products were first implicated in colorectal tumorigenesis back in 1983 when Gardner's syndrome patients, who develop multiple colon polyps, had a reduced adenoma burden after NSAID treatment [123, 129]. This increase in $PGE_2$ is due to aberrant expression of the cyclooxygenase (COX) enzymes COX-1 and COX-2. Deng, Z. *et al* (2015) demonstrated that within the intestine, $PGE_2$ expression was elevated due to enteropathogenic bacteria-secreted particles stimulating the epithelium [207]. It is thought that increased intestinal $PGE_2$ enhances epithelium production of the chemokine ligand CCL2, and these molecules work together to recruit pro-inflammatory Th17 cells and stimulate proliferation, this increased inflammatory environment promotes tumour growth [207].

This has been demonstrated in animal disease models, but is also comparable to what is observed in human colorectal patients, which confirms the importance of the role and mechanism of action of $PGE_2$ on immune cells within organs. Additionally, this demonstrates the role that the gut microbiota can play, affecting $PGE_2$ expression within the intestinal environment, which can consequently modulate the immune response.

Long-term use of non-steroidal anti-inflammatory drugs (NSAIDs), which can suppress $PGE_2$ expression through inhibition of COX-1 and COX-2, can have long-term benefits such as reducing the risk of developing diseases such as Alzheimer's and colorectal tumours as previously discussed [157]. One NSAID is even included on the World Health Organisation (WHO) Model List of Essential Medicine (2015), and widely used by people due to ease of purchase over-the-counter. Nevertheless, for some patients with inflammatory conditions such as inflammatory bowel disease (IBD), administration of COX inhibitors could worsen symptoms, rather than reduce the pro-inflammatory activity within this environment [47, 207].

It was thought that NSAID use, inhibiting both COX enzymes non-specifically, resulted in intestinal inflammation due to reduced levels of COX-1 in the intestine. COX-1 is vital for expression of house-keeping levels of prostaglandins, some of which are important for maintenance of the intestinal barrier as aforementioned. COX-2 specific inhibitors have been designed, as this enzyme is mainly involved in inflammation and pain, also to reduce the risk of gastrointestinal complications because COX-1 is an important in maintaining house-keeping levels of prostaglandins, however, the risk of myocardial infarctions and strokes were increased [208, 209]. This is largely due to suppression of $PGI_2$ that normally induces thrombomodulin production, which reduces the risk of blood coagulation by converting thrombin into an anti-coagulant enzyme [208]. This risk was reduced by use of non-specific NSAIDs, suggesting that use of specific inhibitors to suppress $PGE_2$ production within the intestine had variable results, while non-specific NSAIDs can damage the intestine, leading to a conundrum about treatment options [209]. Additionally, suppression of COX-2 can reduce resolvin production, which could worsen symptoms, although some can be produced by leukotrienes [210]. This may be why an increase in neutrophil markers was also detected in colons of indomethacin-treated mice, due to leukotriene induced recruitment (**chapter 4, Fig. 4.4**).

Specific prostaglandin synthase inhibitors are thought not to be a suitable alternative, as $PGH_2$ can be re-diverted towards another synthase, resulting in other adverse effects [124, 137]. Blocking further up at phospholipase 2 is also not advised, as it is a very diverse enzyme so very tough to develop drugs against, and additionally it would inhibit the production of multiple products vital for housekeeping roles if blocked, therefore more receptor or organ specific treatment is required. Indomethacin was used as the NSAID to inhibit prostaglandin $E_2$ synthesis for the majority of experiments, due to its ease of administration and its use previously by our group and others as an efficient way of inhibiting prostaglandin synthesis [112, 120, 211, 212]. It can inhibit both COX-1 and COX-2, important within the intestine as COX-1 is present for housekeeping prostaglandin production, however inflammation significantly upregulates COX-2 expression, thus an inhibitor of both isoforms can demonstrate the importance of inhibition of $PGE_2$ synthesis. An EP4 agonist was used to confirm that the results observed were due to inhibition of $PGE_2$. Celecoxib, another NSAID which is a specific COX-2 inhibitor, was used by our group to determine whether other NSAIDs had a similar effect on intestinal Tregs (data unpublished). As observed in mice treated with indomethacin, celecoxib treatment also resulted in an increased proportion of intestinal $Foxp3^+$ Tregs, and additionally there was increased RORɣt expression within this Foxp3 expressing population. Celecoxib has similarly been shown in a double-blind study to reduce colorectal adenomatous polyps due to suppression of the overexpressed COX-2 observed in human colorectal cancer and adenomatous polyps [122, 129]. Similarly, an EP4 agonist prevented this increase.

## 7.5 The role of gut microbiota and $PGE_2$ on intestinal Tregs

The gastrointestinal epithelium is constantly exposed to potentially pathogenic stimuli such as the gut microbiota, of which there are approximately ten times more microbial cells in the human intestine than there are cells making up the body and the microbiome contains more than 100 times more genomic material than consists within the human genome, therefore regulatory mechanisms are vital to prevent an unnecessary immune response to a harmless signal [89]. The microbiome does have some benefits for the host, including; regulating the immune system, protection against harmful pathogens, production of energy through fermentation of undigested dietary fibre, and maintaining gut epithelium integrity [213].

Gut microbiota dysbiosis, a microbial imbalance, can result in negative effects, such as allowing pathogenic bacteria to adhere to intestinal epithelium, and altering microbial metabolite products which normally enhance regulatory cell development, thus inflammatory symptoms as observed by IBD patients can develop [214].

The understanding of the importance of gut microbiota has been rapidly increased within the last few years, within both the scientific and general community. IBD patients treated with prebiotics, non-digestible substances that help support growth of the beneficial gut microbiota, and fermented products such as butyrate to provide an energy source for the intestinal epithelium, resulted in reduced IBD symptoms [214]. This demonstrates the importance and 'ease' of modulating the immune system via delivery of dietary supplements which are less severe than current IBD treatments, such as immunosuppression [214]. Probiotic treatments, delivery of live microorganisms to fill a niche and reduce inflammation within the intestine, have been trialled, however due to differences in lengths of therapy and great variety of heterogeneity of microbial specifies, and various patient disease states, have resulted in varying results from trials [214].

It has recently been published that there is evidence that certain gut microbiota can boost cancer therapy, and modulate susceptibility to harmful side-effects [215-219], its importance in regulating blood pressure [220] and involvement in diabetes and obesity [221]. More specific examples are the method of curing *H. pylori* infections with the aim to reduce symptoms of peptic ulcers, and faecal transplants have been used for curing *C. difficile* infections, [222]. These products can signal via APC toll-like receptors, acting via MyD88 and TRIF signal adapters to influence the immune response. Suppression of $PGE_2$ did not affect colonic Treg levels in mice treated with broad-spectrum antibiotics, demonstrating the importance of the gut microbiota facilitates $PGE_2$ mediated inhibition of $Foxp3^+$ Tregs (**chapter 5, Fig. 5.1**). Additionally, MyD88 and TRIF double knock-out mice did not detect an increase in colonic $Foxp3^+$ Tregs following $PGE_2$ inhibition (**chapter 5, Fig. 5.2**). All this just further demonstrates the importance of the intestinal microbiota in development of the immune system, and its potential for its manipulation to be used to benefit the host.

The importance of the gut microbiota and its diversity is clear from birth, and various practices can influence it during the whole lifetime. Studies have indicated that numerous prescribed antibiotics, are unnecessarily [51]. This both increases the risk of developing strains of antibiotic-resistant bacteria, but intestinal microbial dysbiosis can also occur. Microbial dysbiosis is implicated in conditions such as Ulcerative colitis [51, 52, 55, 171]. A greater microbial diversity correlates with a greater population of intestinal Tregs, and this is consequently more protective for the host [55]. More recently, in 2017 a paper was released describing the role that intestinal microbiota plays on cancer patients responses to immunotherapy [223, 224]. Therefore, due to the close link between the microbiota and immune cells, it was considered whether the microbiota also influences $PGE_2$'s suppression of intestinal Tregs.

## 7.6 The involvement of mononuclear phagocytes in intestinal immune homeostasis

My data demonstrated that gut microbiota was involved in $PGE_2$'s suppression of intestinal Tregs, and this was demonstrated via use of broad-spectrum antibiotics and toll-like receptor adapter molecule MyD88/TRIF double knock-out mice. Multiple cell types, such as dendritic cells (DCs), a mononuclear phagocyte (MNP) subtype, are required within the intestine to process microbial peptides and regulate the immune system. $CD103^+$ DCs are known to enhance intestinal Tregs via retinoic acid (RA) and TGFβ production, therefore it was questioned whether inhibition of $PGE_2$ also influenced colonic MNP subtypes. $PGE_2$ is known to suppress differentiation of retinal dehydrogenase, the enzyme responsible for RA synthesis [110]. It was interesting that $PGE_2$ inhibition resulted in increased $CD11b^+CD103^-$ cLP MNPs compared to vehicle treated mice, whereas $CD11b^+CD103^+$ cLP MNPs were unaffected (**chapter 5, Fig. 5.3**).

The importance of these cells within this environment and the link between the gut microbiota was supported by Nakahashi-Oda, C. *et al* (2016) [40]. This group demonstrated that gut microbiota signalling via $CD103^-$ DCs induced IFNβ signalling and consequently enhanced $Foxp3^+$ Treg proliferation [40].

There was an increased expression of type 1 interferon genes, such as *Ifn-β*, in colonic $CD11b^{+}CD103^{-}$ cLP MNPs sorted from indomethacin-treated mice compared to vehicle-treated mice (**chapter 5, Fig. 5.7**). This increased IFN-β expression is thought to enhance colonic Treg proliferation. Nakahashi-Oda. C, *et al* (2016) suggested that CD300a, expressed on $CD103^{-}$ DCs, is activated by phosphatidylserine (PS), a marker expressed on dying epithelial cells and consequently prevents IFNβ production [112]. CD300a is important for preventing Treg proliferation, important within a site of self-renewing epithelial cells which need to be cleared by a variety of phagocytic immune cells. This suggests that the increased type 1 interferon gene expression that was detected is not due to any potential intestinal damage that could result from indomethacin treatment. A greater number of dying epithelial cells would have increased PS expression, and consequently suppressed IFNβ production. The gut microbiota can initiate IFNβ production due to TLR4 activation, which may be why no increase in $ROR\gamma t^{+}$ Tregs are observed in antibiotic-treated or MyD88/TRIF double knock-out mice (**Chapter 5, Fig. 5.1, 5.2**) [6, 50].

## 7.7 The use of disease models to confirm my hypothesis

I have demonstrated the role of $PGE_2$ on intestinal $ROR\gamma t^{+}$ Treg levels and the influence of $CD11b^{+}CD103^{-}$ cLP MNPs and microbiota. Thus, to illustrate the potential pathological importance of the *in vitro* and *in vivo* results, intestinal disease models were used.

In **chapter 4, Fig. 4.6,** it was demonstrated that Foxp3 levels were unaffected in T cell specific EP4 KO mice, although they did have an increased level of RORγt within the Foxp3 positive population. Thus, a DSS colitis disease model was used to see whether the EP KO were more protected from disease induction. Chemical methods of colitis induction, such as DSS and 2,4,6-trinitro benzene sulfonic acid (TNBS) are frequently used by groups to help further understand the mechanisms of cells involved in intestinal inflammation in a short-term disease model [58, 112, 225].

In my DSS colitis disease model, both EP4 KO and WT mice had similar disease scores and inflammatory markers at the end of the experimental period which suggested that the increase in RORɣt expression within the intestinal Foxp3 expressing cell population was not enough to protect from disease induction. This also may have been due to the harshness of the treatment damaging the intestine, therefore an increase in Tregs would not be enough to prevent the damage and inflammatory cell infiltration. If future treatments are wanting to target EP4 receptors, it is necessary to be careful from translation from mouse to human, as $PGE_2$ signalling via EP4 within the colon, can induce production of gastrointestinal bicarbonate to neutralise gastric acid to maintain a balanced pH within the protective mucosal layer of the stomach and duodenum, however in mice the $PGE_2$ receptor EP3 is also able to do this [85]. Thus inhibition of intestinal EP4 in humans will cause intestinal damage by the increased levels of gastric acid inability for neutralisation [85].

T cell colitis was used as a milder disease model to more easily observe the effect of naïve T cells from either wild type or T cell specific EP4 knock-out mice in $RAG^{-/-}$ mice. T cell specific EP4 knock-out mice were shown in previous experiments to have a greater number of $Foxp3^+$ Tregs within the colon compared to WT mice (**chapter 4, Fig. 4.6**). In the T cell colitis disease model, mice injected with EP4 knock-out naïve T cells, had a better disease course and reduced disease symptoms compared to mice injected with WT naïve T cells (**chapter 6, Fig. 6.3**). This may be due to their increased ability to be converted into highly suppressive and stable Tregs within the intestine, as there was an increased proportion of Foxp3 observed in the colonic tissue and reduced inflammatory cytokines compared to mice injected with WT naïve T cells. Due to the size of the flow cytometry fluorophore panel, RORɣt was not able to be also measured within these cells.

A T cell colitis curative disease model using $Foxp3^+$ Tregs sorted from colonic tissue from vehicle- or indomethacin- treated mice was attempted, however the low number of live $Foxp3^+$ Tregs that we collected following the sort was not enough to treat the number of mice needed for a significant result.

Lastly, a faecal transfer model was utilised as earlier *in vitro* work had demonstrated that caecal content from indomethacin-treated mice was able to greater induce Foxp3 expression in naïve T cells compared to vehicle-treated mice caecum (data not shown), and it also boosted $CD103^-$ MNPs ability to induce Tregs (**chapter 5, Fig. 5.5**). Short chain fatty acids, such as butyrate, are produced by bacterial fermentation of dietary fibres, and can inhibit histone deacetylation in the gut, boosting acetylation within the enhancer and promoter regions of various regulatory gene regions, increased acetylation enhances stability of protein levels, important within the intestinal environment [226]. Thus, affecting the gut microbiota present within the intestine, and consequently their metabolite fermentation products, can greatly influence epigenetic regulation of gene expression in cells within this environment [226]. Western blots could be used to detect whether the acetylation state of the *Foxp3* gene within regulatory T cells sorted from colonic tissue from vehicle, indomethacin, or indomethacin plus an EP4 agonist treated mouse, had been affected.

The importance of the gut microbiota and their subsequent metabolic products, was also supported by work by Nakahashi-Oda, C. *et al* (2016) showing that faecal contents promotes IFN-β production from cultured BMDCs *in vitro* [6]. There was no difference in Foxp3 expression observed between naïve T cells cultured with the other subsets of DCs, and as previously discussed, increased levels of IFN-β production increase the proliferative and suppressive ability of Tregs [6]. Faecal transplants have already been used as a method for curing *C. difficile* infections, demonstrating that this is a viable method of treatment [222].

Mice had greater protection from DSS colitis induction after gavage of fresh caecum content solution from indomethacin-treated mice, compared vehicle-treated mice. This was supported by findings from Xiao, X. *et al* (2017), who demonstrated that indomethacin-caecum content protected mice against indomethacin-induced injury [195]. This was shown to be largely due to the alteration of the gut microbiota phyla [195]. There was an increased abundance of Firmicutes in the gut of indomethacin treated mice and a reduced level of Bacteroidetes, compared to mice treated with vehicle [195]. However $PGE_2$ and COX levels were similarly reduced in both of the indomethacin-injury induced groups of mice treated with either the vehicle or indomethacin treated mouse faecal content, demonstrating the importance of the gut microbiota in the protection of the intestine [195]. In **chapter 6, figure 6.5**, fresh caecum content from indomethacin treated mice had a protective benefit in DSS colitis induced mice compared to the mice gavaged with caecal content from vehicle-treated mice. Like data observed in Xiao, X *et al* (2017), they had a reduced disease activity score and lower inflammatory cytokines present in colonic tissue from mice given faecal transplants from indomethacin treated mice.

Other experiments that could be used to further confirm our microbiota data is a co-housing experiment where marked mice are gavaged with either vehicle or indomethacin and co-housed with untreated mice. Induce a colitis disease model and observe whether mice are protected from disease if co-housed with mice treated with the NSAID. Further suggested experiments include a faecal transfer model into germ-free (GF) mice from vehicle or indomethacin treated, to compose a microbiome, and induce T cell colitis, to see whether a microbiome from indomethacin-treated mice has a protective benefit. Additionally, it would be useful to phenotype the gut microbiota in stored caecum content from the two donor groups using RNA based sequencing and PCR to see whether the taxonomic composition of microbiome had been increased, and whether any bacterial phyla, or more specifically genera, had been reduced during treatment, and to see whether alteration had also been observed in the recipient mice post-transfer.

There was increased type 1 interferon gene expression detected in sorted colonic $CD103^-$ mononuclear phagocytes (MNPs) from indomethacin treated mice, compared to vehicle treated mice (**chapter 5, figure 5.7**). Additionally, there was also increased STAT-1 signalling, a downstream marker of interferon signalling, detected by increased levels of phosphorylation, in both colonic $Foxp3^+$ Tregs and MNPs (**chapter 5, Fig. 5.9**). Multiple groups have demonstrated that inducing the type 1 interferon signalling pathway via TLR signalling or interferon agonists resulted in a reduction of DSS colitis severity [76, 227-230]. Thus, the faecal transplant of caecal content from indomethacin treated mice may have induced increased colonic TLR signalling in the recipient mice and hence reduced colitis symptoms [76, 227-230]. This may be due to the importance of protection against epithelial damage, which is a concern when inhibiting $PGE_2$ directly using NSAIDs and negates much benefit from treatment, whereas type 1 interferons has been shown to inhibit epithelial apoptosis [76, 229].

Genome studies of IBD patients have identified changes in IFNRA1 and subsequent downstream signalling molecules including STAT-3, as disease risk loci, demonstrating the importance in type 1 interferon in controlling intestinal inflammation [76]. The commensal microbiota are key in establishing a basal type 1 interferon response, which then can control the level of immune response with the intestine demonstrating the importance of the gut microbiota in regulation of the immune system [76]. Additionally, MyD88 and TRIF signalling can influence the interferon response in intestinal epithelial cells (IECs) within the colon, and previously we had demonstrated the importance of these signalling pathways in $PGE_2$'s modulation of colonic Treg or DC levels (**Chapter 5. Fig. 5.2 and 5.4**) [76]. Bliech. A, *et al* (2009), demonstrated that addition of a CpG-oligodeoxynucleotide, a molecule that mimics bacterial signalling and can induce interferon signalling, induced Tregs and protected mice from T cell colitis [231]. This was dependent on type 1 interferons and TGF-β signalling, both negatively affected by $PGE_2$ signalling [76, 87, 231]. This signalling can also retain T cells in the mucosal area, consequently increasing exposure to microbial antigens [76].

Further evidence demonstrating the importance of IFNβ was demonstrated by Kole, A. *et al* (2013) who injected $RAG^{-/-}IFNAR^{-/-}$ DKO, deficient of both mature B and T cells, and interferon receptors, inhibiting interferon signalling, or $RAG^{-/-}$ KO mice with $CD4^{+}CD45RB^{hi}$ T cells, and observed less severe symptoms in the $RAG^{-/-}$ mice, due to their ability to signal via the IFN pathway [75]. The suppressive ability of interferons results in reduced effector T cell proliferation. There is restricted data in human studies, however clinical trials treating IBD patients directly with IFN-1 was not highly successful [75, 232]. Whereas the role of interferon signalling and the commensal microbiota is becoming a widely more studied area and has promising results for future reduction of human conditions of intestinal inflammation rather than use of NSAIDs or direct administration of interferons as the levels and balance of these can affect both the pro- and anti-inflammatory immune response.

Lastly, a stimulating concept was introduced by Professor Glenn Gibson at an Society of Chemical Industry (SCI) lecture titled 'Getting to the gut of microbiology' [233]. It was suggested that in the future rather than using antibiotics to combat bacterial infection, risking negatively affecting the intestinal gut microbiota and causing antibiotic-resistant bacteria, phages will be developed to bind to specific bacteria and kill it [233]. Therefore, in the future, rather than using broad-spectrum antibiotics to influence intestinal microbiota, selectively killing pro—inflammatory bacteria could allow regulatory bacteria to fill the remaining niche. This would be able to benefit the 300,000 people in the UK who have IBD (numbers taken from the UK Inflammatory Bowel Disease Audit by the Royal College of Physicians in 2014) [116]. The body of work presented here indicates the influence $PGE_2$ has on colonic Tregs and the impact this could have on intestinal inflammation, potentially through altering the intestinal microbiome. This also introduces the concept of using modulation of intestinal $PGE_2$ to selectively foster anti-inflammatory, regulatory microbiota as part of a multifaceted treatment regime to tackle gut problems. These problems are reported to take up more GP time than any other complaint, and therefore this work may ultimately contribute to more effective relief for patients with these pathologies [233].

## 7.8 Conclusion

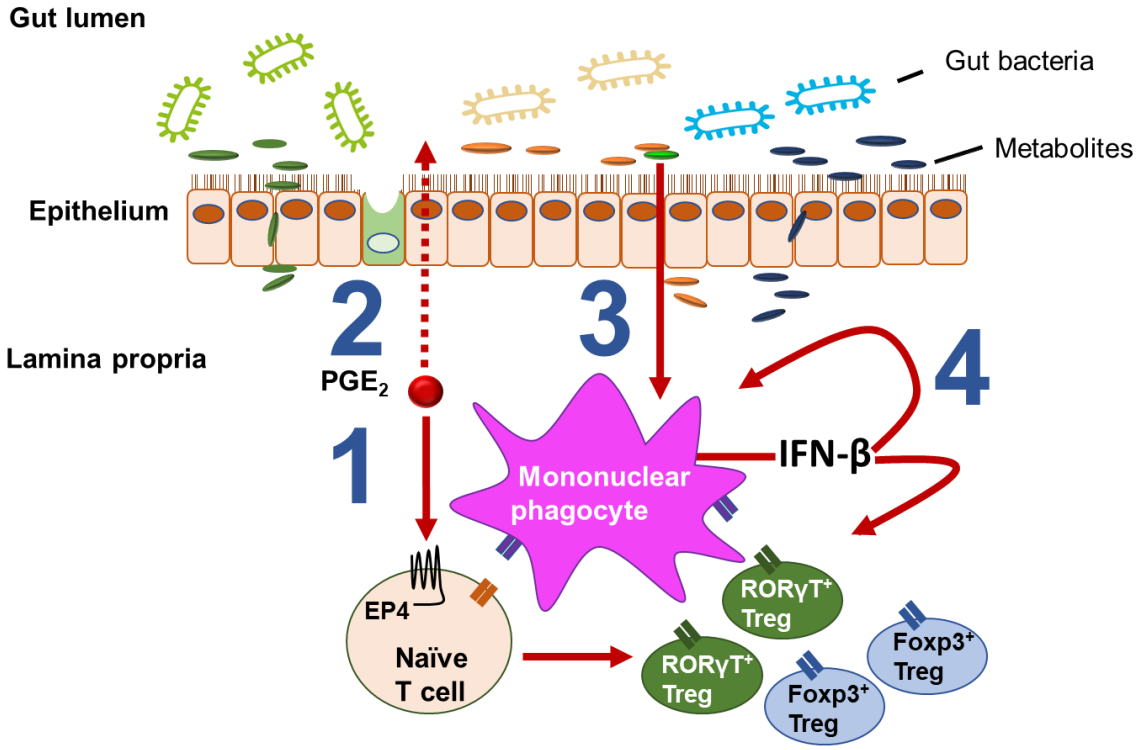


**Figure 7.1: Summary of the role of $PGE_2$ on intestinal Treg development.** *(1) $PGE_2$ acts via the EP4 receptor to negatively affect naïve T cell differentiation into Foxp3$^+$ Tregs. This was observed to the greatest extent within the colon. (2) $PGE_2$ can modulate the gut microbiota which consequently influences both Foxp3$^+$ Tregs and Foxp3$^+$RORɣt$^+$ Treg differentiation in the colon through detection by TLRs and the adapter molecules MyD88 and TRIF. (3) Modulation of the gut microbiota can also affect intestinal CD103$^-$ mononuclear phagocyte development, which consequently affects colonic Foxp3$^+$ Tregs and Foxp3$^+$RORɣt$^+$ Tregs. (4) Inhibition of $PGE_2$ results in increased type 1 interferon gene expression in colonic CD103$^-$ mononuclear phagocytes, which can increase induction of intestinal Foxp3$^+$ Tregs. Inhibition of type 1 interferon signalling prevents an increase in colonic CD103$^-$ mononuclear phagocytes, Foxp3$^+$ Tregs, co-expressing Foxp3$^+$RORɣt$^+$ Tregs after inhibition of $PGE_2$.*

In conclusion, this thesis has introduced novel concepts in regard to how the well-known inflammation mediator prostaglandin $E_2$ modulates intestinal Tregs, especially the RORɣt$^+$ subset. **Figure 7.1** illustrates the model I propose to explain. This is possibly through modification of the gut microbiota which consequently influences colonic mononuclear phagocyte subsets, and these findings can potentially help further enhance research into intestinal inflammation and development of more specific, less severe therapeutic strategies for treating inflammatory bowel disease.

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# 9 Appendix

## 9.1 Papers published

- Duffin, R., et al., *Prostaglandin E(2) constrains systemic inflammation through an innate lymphoid cell-IL-22 axis.* Science, 2016. **351**(6279): p. 1333-8.
- Robb, C.T., et al., *Prostaglandin E2 stimulates adaptive IL-22 production and promotes allergic contact dermatitis.* J Allergy Clin Immunol, 2017.
- Felton, J.M., et al., *Facilitation of IL-22 production from innate lymphoid cells by prostaglandin E.* Thorax, 2018.
- Crittenden, S., et al., Purine metabolism drives innate lymphoid cell mediated protection against intestinal inflammation. In progress.

## 9.2 Oral presentations

- **Research In Progress talk** (February 2016) Edinburgh University. Talk entitled 'Prostaglandin $E_2$ in Regulatory T cell Development.'
- **Research In Progress talk** (December 2016) Edinburgh University. Talk entitled 'Prostaglandin $E_2$ in Regulation of Intestinal Regulatory T cells'.
- **Young Lipid Scientist Award meeting** (June 2016). London SCI. Talk entitled 'Prostaglandin $E_2$ and the Development of Intestinal Regulatory T cells'.
- **Young Lipid Scientist Award meeting** (June 2017). London SCI. Talk entitled 'Prostaglandin $E_2$ inhibits Regulatory T cells through Gut Microbiota'.

## 9.3 Poster presentations

- **Edinburgh Immunology Group Summer Symposium** (June 2017) Edinburgh.
- **4th European Congress of Immunology** (September 2015) Vienna.
- **10th European Mucosal Immunology Group Meeting** (October 2016) Copenhagen.